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DIFFERENTIAL EFFECTS OF MALEIC HYDRAZIDE ON THE GROWTH OF LEAF AND STEM RUSTS OF WHEAT[1]

D. J. SAMBORSKI, CLAYTON PERSON, AND F. R. FORSYTH[2]

Abstract

The effect of maleic hydrazide on leaf rust and stem rust of wheat was investigated, using intact plants supplied with maleic hydrazide through the roots, detached leaves floated on solutions of maleic hydrazide, and spores germinated on maleic hydrazide solutions in agar. In each case the growth of leaf rust was inhibited at concentrations of maleic hydrazide which had no effect on the growth of stem rust. Some resistant wheat varieties became susceptible to rust after treatments with levels of maleic hydrazide which were not inhibitory to rust development. In some experiments, leaf rust races reacted differentially to maleic hydrazide. Both nutritional and inhibitory factors could be involved in resistance and host–pathogen specificity.

Introduction

Maleic hydrazide, a growth inhibitor of higher plants, has been used in a number of studies of host:parasite relations (3, 4, 8, 9, 12). A general result, with stem rust of wheat, is that resistant plants become less resistant when maleic hydrazide is taken up through the roots. With leaf rust of oats (crown rust), Simons found that susceptible plants, previously sprayed with maleic hydrazide, did not become infected (16). However, Simons did observe a few pustules of a more susceptible type on plants of two of the resistant varieties that were treated. Since these varieties were inoculated after treatment, it is possible that spore germination was inhibited by the treatment. It is also possible that the leaf and stem rusts of cereals differ in their responses to maleic hydrazide. In this investigation the effects of maleic hydrazide on the leaf and stem rusts of wheat were studied.

Methods

For studies of rust development on intact plants, the maleic hydrazide was supplied through the roots to plants that were potted in Ottawa quartz sand. For studies with detached leaves, the methods previously described (11) were used, and the maleic hydrazide was added to the culture solutions. For studies of spore germination and hyphal growth, the maleic hydrazide solutions were

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Contribution No. 24 from the Canada Department of Agriculture Research Station, Winnipeg, Manitoba.
[2]Plant Pathology Laboratory.

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prepared in 1% agar, and a group of 5-cm petri dishes, comprising an entire set of dilutions of maleic hydrazide, was placed under a large bell jar. Rust spores of a single race were puffed into the bell jar, where they settled uniformly over all the dishes. After incubation the germinated spores were fixed and stained, using Isaac's method (7). Measurements were taken from 100 spores of each treatment.

Results

The effect on rust development of maleic hydrazide, supplied through the roots to intact plants, is shown in Table I. Maleic hydrazide applied daily, starting 7 days before inoculation, had no effect on the development of the stem rust on Little Club, which is a susceptible variety, even at the highest concentration used, but at 10 and 100 mg per day it increased the susceptibility of Khapli, which is normally a resistant variety. At 100 mg per day, maleic hydrazide inhibited the development of leaf rust on both varieties. In all cases, whether the maleic hydrazide was supplied at 10 or 100 mg per day, there was a marked inhibition in growth of the host plants.

TABLE I

The effects of maleic hydrazide on stem rust and leaf rust reactions in Khapli and Little Club wheats*

Treatment, mg of M.H./day	Khapli		Little Club	
	Stem rust	Leaf rust	Stem rust	Leaf rust
0	1−	3+	4	4
10	2,3	4	4	4
100	3	0;	4	0;

*Treatments started 7 days before inoculation. Stem rust, race 15B; leaf rust, race 15. Rust reactions according to Stakman *et al.* (17).

Experiments were carried out to determine the effect on rust development of treating the intact host plants with maleic hydrazide for different periods of time before inoculation. The results, summarized in Table II, show that with increasing concentrations of maleic hydrazide, a shorter preinoculation treatment induced susceptibility to stem rust in the variety Khapli. With preinoculation treatment of 100 mg per day, both Little Club and Khapli quickly became resistant to leaf rust.

The effect of maleic hydrazide preinoculation treatment on the reaction of a few variety:race combinations, in which differing degrees of resistance to leaf rust are expressed, are shown in Table III. It is seen that those varieties with greatest resistance remained resistant, while those with less resistance became more susceptible after moderate preinoculation treatment with maleic hydrazide.

TABLE II

Development of stem rust (race 15B) and leaf rust (race 15) on Khapli and Little Club wheats after different conditions of treatment with maleic hydrazide

Treatment		Khapli		Little Club	
Mg of M.H./day	Preinoculation interval, days	Stem rust	Leaf rust	Stem rust	Leaf rust
0	3	1−	3+	4	4
0	6	1−	3+	4	4
0	9	1−	3+	4	4
0	12	1−	3+	4	4
2.5	3	1−	3+	4	4
2.5	6	1−	4	4	4
2.5	9	1−	4	4	4
2.5	12	1,2	4	4	4
10	3	1−	4	4	4
10	6	1,2+	4	4	4
10	9	2,3	3+	4	4
10	12	3	3	4	4
100	3	1,2	1,2	4	X−
100	6	3	0;	4	0;
100	9	3+	0;	4	0;
100	12	3+	0;	4	0;

TABLE III

Effect of maleic hydrazide on leaf rust reaction

Variety	Leaf rust race	Rust reaction	
		Untreated	Treated*
Colotana (266/51)	15	0;	0;
Rio Negro	15	0;	0;
Webster	15	0;	0;
Democrat	11	1	X
Hussar	58	1+	3+
Selkirk	15	2	3+

*Ten milligrams maleic hydrazide per day, begun 7 days before inoculation.

Differences in response of leaf and stem rust infections, obtained after supplying maleic hydrazide through the roots to intact plants, were also obtained on detached leaves when maleic hydrazide was added to the culture solutions. With detached leaves the levels of treatment can be controlled more accurately. In all studies the leaves were inoculated 1 day before treatment, in order to avoid any possible effect of the treatment on spore germination. The difference in the response of leaf and stem rusts to maleic hydrazide treatment is shown very clearly in Table IV, where it is seen that concentrations of maleic hydrazide that have little effect on stem rust development on the variety Little Club are able to inhibit completely the development of leaf rust on this variety.

TABLE IV

Growth of stem and leaf rusts of wheat on detached leaves of Little Club wheat floated on various levels of maleic hydrazide

Treatment, M.H. (p.p.m.)	Rust development				
	Stem rust races		Leaf rust races		
	15B	56	1	5	30
0	Heavy	Heavy	Heavy	Heavy	Heavy
50	Heavy	Heavy	Heavy	Heavy	Heavy
75	Heavy	Heavy	Slight–moderate	Moderate	Moderate
100	Moderate	Moderate	Slight	Slight	Slight
125	Moderate	Moderate	Trace	Slight	Trace
150	Moderate	Moderate	0	Slight	0
175	Moderate	Moderate	0	Trace	0
200	Moderate	Moderate	0	0	0
250	Moderate	Moderate	0	0	0
300	Slight–moderate	Slight–moderate	0	0	0
350	Slight	Slight	0	0	0
400	Trace	Trace–slight	0	0	0
450	Trace	Trace	0	0	0
500	0	0	0	0	0

*All treatments contained 30 p.p.m. benzimidazole.

In other experiments, where reactions of 10 different leaf rust races were compared, there were indications that different races of leaf rust varied in their responses to maleic hydrazide treatment. However, even though clear-cut differences were obtained in many single experiments, the experimental results were not readily reproducible. The reasons for this variability are not known. The effect of leaf age at the time of inoculation was investigated, but no conclusive results were obtained. Leaf rust on older leaves was more sensitive than on younger leaves to maleic hydrazide in some experiments; in other experiments the reverse was obtained.

TABLE V

Effects of maleic hydrazide on spore germination and germ tube length in stem and leaf rusts of wheat

Treatment, M.H. (p.p.m.)	Percentage germination			Average germ tube length, μ		
	Stem rust, race 15B	Leaf rust		Stem rust, race 15B	Leaf rust	
		Race 1	Race 11		Race 1	Race 11
0	97	96	94	208	213	206
50	97	96	98	202	185	202
75	98	83	93	70	81	74
100	98	87	95	75	102	103
125	95	34	82	34	58	53
150	100	26	79	17	43	46
175	91	34	70	23	40	47
200	96	22	58	16	37	45
250	45	2	27	10	10	19
300	41	0	7	10	0	14
350	34	0	17	10	0	14
400	23	0	9	10	0	11
500	0	0	0	0	0	0

Differences in response of leaf and stem rusts to maleic hydrazide were also obtained in studies on spore germination. The results of a typical experiment are shown in Table V. Spores were incubated for 4 hours and the percentage germination and average germ tube lengths of germinated spores were determined. While the two leaf rust races used differed in their responses, spores of both races were more sensitive to maleic hydrazide than were spores of stem rust.

Longer incubation periods were tested to determine whether maleic hydrazide merely reduced the rate of spore germination and germ tube elongation. It can be seen from Table VI that increasing the incubation period from 4 to 24 hours did not lead to any increase in percentage germination or germ tube elongation.

TABLE VI

Response of leaf rust spores to different conditions of treatment with maleic hydrazide

Treatment		Percentage spore germination		Average germ tube length, μ	
Maleic hydrazide, p.p.m.	Incubation period, hours	Race 1	Race 11	Race 1	Race 11
0	4	100	100	340	410
0	13	100	100	*	*
0	24	100	100	*	*
100	4	63	92	65	98
100	13	71	92	60	83
100	24	83	85	64	74
200	4	29	59	40	61
200	13	29	61	40	47
200	24	25	59	32	41
400	4	0	0	0	0
400	13	0	5	0	35
400	24	0	5	0	35

*Longer than those in 0 p.p.m. for 4 hours; accurate measurements were not taken.

Discussion

This investigation shows clearly that the results of studies with maleic hydrazide on leaf and stem rusts of wheat will be affected by the method of application and the concentration used. Maleic hydrazide applied to the leaves before inoculation can inhibit germination of rust spores. Low concentrations of maleic hydrazide applied to the roots would tend to increase susceptibility of wheat plants to leaf and stem rusts while higher concentrations would inhibit the growth of leaf rust.

The nature of resistance and susceptibility to disease, and of host–pathogen specificity, are among the most intriguing problems in plant pathology. The apparently complex pattern of host–pathogen specificities, for example interactions of wheat differential varieties and the innumerable rust races, is

made up of numerous individual resistant or susceptible reactions. Biochemically, therefore, these problems are identical, and with any particular host and pathogen, the biochemical explanation of resistance and susceptibility would also be the explanation of the specificity displayed.

Although a number of schemes have been advanced by workers on cereal rusts, there is still no detailed explanation for the resistance or susceptibility of any one variety to a race of rust. Recent suggestions have emphasized qualitative and quantitative nutritional relationships (4, 11, 12, 15), effects of hormone metabolism on such relationships (14), and the dual action of inhibition and nutrition (5).

Our studies have shown that a consistent feature associated with induced susceptibility of wheat plants is the accumulation of soluble nutrients in the leaf (4, 11, 12). A similar result is obtained with maleic hydrazide as a consequence of growth inhibition (4, 13). However, above a critical level in the leaf, maleic hydrazide has a direct inhibitory effect on the pathogen. In fact, a range of sensitivity to maleic hydrazide is shown in this study. For example, whereas the growth of wheat plants is inhibited by treatment with 10 mg per day of maleic hydrazide, both leaf and stem rusts grow rapidly in the leaves of these treated plants, and on certain normally resistant varieties rust growth is much improved after treatment. Treatments with 100 mg per day inhibit the growth of wheat plants and of leaf rusts but have no effect on stem rust. Clearly the order of sensitivity is wheat plants > leaf rust > stem rust. Had the rusts proved more sensitive than wheat plants to maleic hydrazide, treatment could not have resulted in decreased resistance, and indeed, maleic hydrazide would act as a fungicide.

This study indicates that both nutritional and inhibitory factors could be involved in resistance and specificity, which would be in conformity with the nutrition–inhibition hypothesis proposed by Garber (5). The induced susceptibility of some varieties treated with maleic hydrazide is of obvious significance in studies on host–parasite relations. However, the differential response of leaf and stem rusts to maleic hydrazide may be of equal importance. Unfortunately, the precise action of maleic hydrazide on higher plants or on the cereal rusts is not known. It has been suggested that it acts on the indoleacetic acid system (10), as an antigibberellin (2), and by inhibiting sulphydryl groups (6). Perhaps it acts in different ways, depending on its concentration and the type of tissue concerned (1). With the current intense interest in growth substances, further information relating to the action of maleic hydrazide will undoubtedly be forthcoming.

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STUDIES ON THE VIRUS-STREAK SYNDROME IN TOMATOES[1]

BLAIR H. MACNEILL AND HIKMET ISMEN

Abstract

The streak disease of tomatoes in Ontario has been shown to be double-virus in nature, resulting from the synergistic action of tobacco mosaic virus (TMV) and potato virus X (PVX). Expression of the disease was found to be a function of both virus components of the complex, a variation in strain characteristics of either TMV of PVX being reflected in the intensity of their interaction in the tomato.

The extreme severity of streak in the greenhouse and field tomato crops in Ontario is associated with what appears to be a geographic strain of TMV. This virus, designated as PLV, is characterized by the induction of primary local lesion in petunia without subsequent systemic invasion of that host.

Air temperature profoundly influences the development of streak, symptoms being masked above the critical temperature of 26° C. This phenomenon is a true masking effect with both viruses becoming latent in the tomato host.

Introduction

The virus-streak disease of tomatoes is widely distributed in both the field and greenhouse crops of Ontario. It is characterized by its sudden onset which often results in the complete devastation of the plants affected.

For the most part the disease is marked by the presence of necrotic lesions on the stem, petioles, leaves, and fruits. On the stem the lesions take the form of dark, longitudinal streaks extending through the cortex and into the pith beneath. Often the leaves show necrotic spots which enlarge and blight the foliage. Fruits may be affected while still green and develop irregular, sunken, necrotic blotches.

The first report of the disease for Canada was made by Howitt and Stone (10), who collected it early in 1915 from a greenhouse in the Hamilton district of Ontario. At that time the virus nature of the disturbance was not appreciated, and attempts were made to obtain control through the application of fertilizers to the tomato crop. Jackson (11) working at Purdue reported in 1917 that the streak disease could be induced in plants after inoculating them with the extracted juice of diseased plants, thus suggesting the virus nature of the syndrome. The first critical study upon the etiology of tomato streak, however, was made by Vanterpool (22), who very ably demonstrated that the syndrome was due to the combined action of two viruses which he referred to as potato mosaic virus and tomato mosaic virus. Subsequent reports (13, 18) have generally supported the evidence brought forth by Vanterpool, although Doolittle (8) has reported that, in the greenhouse crop at least, tomato streak may be caused by the combination of tobacco mosaic virus and cucumber mosaic virus.

[1]Manuscript received September 15, 1959.
Contribution from the Department of Botany, Ontario Agricultural College, Guelph, Ontario.

There is also evidence that the streak syndrome may be the result of the action of but a single virus. Berkeley (4) reported fully upon the epiphytology of a streak disease prevalent in the Niagara Peninsula of Ontario and indicated that the etiological agent was a strain of the tobacco mosaic virus. A similar explanation of tomato streak has been reported by such workers as Ainsworth (1), Jarrett (12), Ainsworth *et al.* (2), and Berkeley and Phillips (6). Newton (16) and Newton and Edwards (17) offered evidence that in British Columbia streak may be caused by a virus of the potato mosaic group unassociated with tobacco mosaic virus, while in England and in Australia the disease has been shown by such workers as Brittlebank (7), Bald and Samuel (3), and Smith (19) to be due to the tomato spotted-wilt virus.

The main purpose of the present work was to investigate the etiology of the tomato streak disease in Ontario; however, some factors which appeared to be important in affecting the expression of the disease in tomato have also been considered.

Materials and Methods

For the most part this study has been concerned with two streak cultures, one collected from a greenhouse tomato at London, Ontario, during mid-winter 1956, and the other from a field tomato collected at Harrow, Ontario, in the summer of 1957. Throughout the course of these investigations these two streak cultures, designated at 34B and 18A respectively, were maintained in tomato plants in 4-in. pots containing a steam-sterilized mixture of two parts soil, one part sand, and one part peat moss. Such plants were kept under conditions of 15 hours daylength, at an air temperature of 21° C in a controlled-climate chamber illuminated with 10 incandescent bulbs and 24 fluorescent tubes which gave a total illumination of approximately 800 ft-c at bench level (15). Other virus cultures such as Johnson's strain of tobacco mosaic virus, the Rothamsted strain of tobacco mosaic virus, and various isolates of potato virus X were maintained in appropriate host plants under similar conditions in controlled-climate chambers.

All inoculations of test plants were made by mechanical means. Juice was expressed from diseased tissue with a mortar and pestle, diluted approximately 1:50 with tap water, then applied to the carborundum-dusted leaves of test plants by rubbing them with a pestle or a cotton swab.

Experimental

Approximately 95 samples of tomato plants exhibiting symptoms of the streak disease were assayed for virus content on appropriate indicator plants. These samples, collected over a period of three seasons from both field and greenhouse crops in Ontario, all induced streak when inoculated to tomato. However, some differences in the intensity of the reaction on certain of the indicator plants suggested that the viruses in the samples were not identical. Accordingly, two streak isolates, designated as 34B and 18A, were selected

for detailed study. On tomato these two isolates represented the extremes of reaction, 34B being very severe and giving rise to rapid terminal necrosis of the inoculated plant, while 18A was slow in its action and relatively mild in the symptoms induced.

Streak in tomatoes, as noted earlier, may be due either to the combined action of two viruses, e.g. tobacco mosaic virus and potato mosaic virus (potato virus X), or to one virus acting alone, e.g. tobacco mosaic virus. In the case of the Ontario collections of streak, there were strong indications, from the preliminary analyses reported above, that the syndrome was the result of the joint action of at least two viruses; accordingly, experiments were aimed at determining the specific nature of the virus-streak complex. For this purpose two techniques were employed: (*a*) the inoculation of differential host plants which might react so as to restrict the movement of some viruses and permit the systemic spread of others; (*b*) the selective action of heat upon plant extracts containing the virus complex.

Analysis of the Streak Complex Using Differential Hosts

The identification of a virus, in so far as its properties as an etiological agent of a disease syndrome are concerned, is in large part dependent upon the specific response incited in a series of differential hosts or indicator plants. The degree of susceptibility or resistance or even immunity of the test plant often indicates the exact virus or group of viruses involved.

In view of the broad spectrum of viruses reported as etiological agents of streak, the analysis of the Ontario streak collections was carried out on a series of differential hosts consisting of 10 plants: *Cucumis sativus* L. var. A & C (cucumber); *Datura stramonium* L. (datura); *Gomphrena globosa* L. (gomphrena); *Lycopersicon esculentum* Mill. var. Bonny Best (tomato); *Nicotiana glutinosa* L. (glutinosa); *Nicotiana tabacum* L. var. Havana 38 (tobacco); *Petunia hybrida* Vilm. var. Rosy Morn (petunia); *Phaseolus vulgaris* L. var. Pinto (bean); *Pisum sativum* L. var. W. R. Perfection (pea); *Vigna*

TABLE I

Response of differential hosts to streak inoculum

Differential host	Primary symptoms on inoculated leaves within 2 to 7 days	Secondary symptoms within 5 to 20 days
Tomato	Yellowish spots turning to brown necrosis	Severe streak on all parts
Cucumber	Nil	Nil
Datura	Local	Systemic mottle and severe necrosis
Gomphrena	Local lesions	Nil
Glutinosa	Local lesions	Severe systemic mottle
Tobacco	Yellowish spots and clearing of veins	Systemic mottle
Petunia	Local lesions	Nil
Pea	Nil	Nil
Bean	Local lesions	Nil
Cowpea	Nil	Nil

sinensis Savi var. Black (cowpea). These plants were inoculated with juice expressed from tomato-streak specimens 34B and 18A and the responses noted after a period of incubation which ranged from 4 days to 4 weeks, depending upon whether infection was expressed only as a primary symptom or later became systemic. On the basis of the responses of these plants (Table I) certain conclusions could be drawn as to the probable virus components of the inoculum. For example, the negative reactions of cucumber and cowpea precluded the existence of cucumber mosaic virus, while the positive reactions of glutinosa and gomphrena were interpreted as indicating the presence of tobacco mosaic virus and potato virus X.

Thermal Differentiation of the Components of the Streak Complex

The results from a study of infection on a range of differential hosts, as reported above, while suggesting the presence of certain viruses and virus strains, did not provide conclusive information as to the identity of the components of the streak complex. Accordingly, a second experimental approach, that of the thermal treatment of extracts from infected plants, was used to ascertain more specifically the nature of the viruses involved.

For this purpose tomato plants growing in a controlled-climate chamber were selected for uniformity at 6 weeks of age, divided into two groups, and then inoculated with the juice from streak-tomatoes using sources 34B and 18A respectively. As soon as streak symptoms appeared on the youngest parts of the inoculated plant, the very top leaves were excised with a sterile scalpel and forceps, and their juice expressed by means of a mortar and pestle. This extract was then pipetted into 3-ml glass vials fitted with air-tight screw caps, and the vials immersed in a water bath which was maintained at the appropriate temperature for a 10-minute interval. Temperatures used in these heat-inactivation tests were selected on the basis of literature reports (20, 9) concerning the thermal differentiation of viruses reported to be etiological agents of streak. Following the thermal treatment, the vials were cooled rapidly in running tap water and the juice used as inoculum on test plants. Inoculated plants were then returned to the controlled-climate chamber and incubated at 20° C for an appropriate period of time. The effect of these thermal treatments upon infective extract is recorded in Table II, in which the reactions of inoculated test plants are described. These reactions were consistent throughout five series of such tests. An examination of this table reveals that inactivation of those components of the complex which were necessary for the induction of streak in tomato did not occur until the temperature of the water bath had reached 68–70° C. This then indicates that the tomato spotted-wilt virus, the tomato blackring virus, and the cucumber mosaic virus were not present in the infective juice since all of these viruses are recorded as having thermal inactivation points considerably below this range. Accordingly, these viruses could not be considered as etiological agents in the streak cultures under study. On the other hand, since the juice was inactivated only in part at 68–70° C, but *in toto* at 89–95° C, it may be concluded

TABLE II

The differential effects of temperature upon juice extracted from streak tomato plants

Temp., °C	Reaction of indicator plants after incubation at 20° C							
	Tom.	Glut.	Dat.	Cuc.	Gomph.	Pet.	Cowpea	Tob.
45	Streak	LL,* MM†	LL	—	LL	LL	—	MM
55	Streak	LL, MM	LL	—	LL	LL	—	MM
65	Streak	LL, MM	LL	—	LL	LL	—	MM
68–70	MM	LL	LL	—	—	LL	—	MM
72–78	MM	LL	LL	—	—	LL	—	MM
82	MM	LL	LL	—	—	LL	—	MM
95	—	—	—	—	—	—	—	—

*LL = local lesions.
†MM = mosaic mottle.

that the streak complex consists of two distinct viruses. These two viruses on the basis of their properties of thermal resistance appear to be within the groups of potato virus X and tobacco mosaic virus, respectively.

In summary of the analyses of the tomato-streak complex as presented in Tables I and II, the following should be noted.

(*a*) The production of systemic symptoms in glutinosa and primary necrotic lesions in gomphrena by a virus which is inactivated at 68–70° C indicates that potato virus X is functioning as an etiological component of the complex.

(*b*) The production of primary necrotic lesions in glutinosa by a virus which is inactivated at 89–94° C suggests that the complex contains a second component which is a virus of the tobacco mosaic group. The additional features whereby this virus induces a local lesion response on the inoculated leaves of petunia, yet fails subsequently to become established systemically in the petunia host, appears to be unique for the strains of TMV previously described.

(*c*) There is no suggestion on the basis of the reaction in tomato or the other hosts of the differential series that streak is due to the action of but a single virus, or that the components of the complex may be other than strains of TMV and PVX.

A Comparison of the Petunia Local Lesion Virus With a Typical Strain of the Tobacco Mosaic Virus

The production of local lesions on petunia in response to virus infection has been reported as a fairly specific reaction to the tomato spotted-wilt virus. However, the occurrence of this type of reaction after a thermal treatment of plant extracts which would eliminate the latter low-temperature virus precluded its existence in the inoculum under study. Rather, evidence from the tests described above suggested that the petunia local lesion virus (PLV) was closely related to the tobacco mosaic virus (TMV). Accordingly, a comparative study of PLV and a typical strain of TMV was initiated. For these purposes Johnson's strain of tobacco mosaic virus (TMV (Johns.)), supplied through the courtesy of Dr. F. O. Holmes, was used as the "typical strain".

TABLE III

Properties of the viruses

Virus	Infectious after 3 months	Not infectious after 10 min at 92° C but infectious at 88° C	Dilution end point
TMV (Johns.)	+	+	10^{-6}
PLV	+	+	10^{-6}

Properties

The results of certain tests carried out in parallel series on the two viruses under study are summarized in Table III. Thermal inactivations were carried out using undiluted tomato extracts according to the technique described previously; resistance to aging was determined by the inoculation of the local lesion hosts glutinosa and petunia with plant extracts which had been stored undiluted at 20° C in vials loosely plugged with cotton; dilution end points were determined by the addition of distilled water to the expressed juice of infected tobacco plants just prior to using it as inoculum on appropriate indicator plants.

Symptoms Induced on Test Plants

For these studies the representative viruses were first freed from possible contaminants by heating them almost to their inactivation points. They were then cultured in young tobacco plants and these subsequently served as a source of inoculum for the test plants glutinosa, petunia, datura, tobacco, and tomato.

In all respects, except for the reaction on petunia, PLV and TMV (Johns.) induced nearly identical symptoms on the test plants. On petunia, however, TMV (Johns.) produced no primary symptoms on the inoculated leaves but became systemic within 7 days of inoculation. PLV, on the other hand, always produced a primary local lesion reaction on petunia but remained restricted to the inoculated parts of the plant.

It would appear on the basis of the reactions of these test plants that the behavior of PLV and TMV (Johns.) on the petunia host serves to differentiate the two virus strains.

Cross Protection

A series of cross protection tests was carried out on petunia, tobacco, and tomato using TMV (Johns.) as the protecting virus against the challenging PLV virus. In all instances the first virus was inoculated mechanically to the host, and after a period of incubation in a controlled-climate chamber at 21° C to allow for systemic invasion of the plant, the second virus was applied.

Experiment 1. Petunia.—Expressed juice from tobacco infected with TMV (Johns.) was used as inoculum for petunia; following this, the test plants were incubated for 15 days at 21° C, at the end of which time the virus had invaded systemically. Inoculum from tobacco containing PLV was then rubbed on the leaves of the already infected petunias and a further 2-week period of incubation allowed to elapse. No local lesions developed as normally occurs when PLV invades its petunia host. It was concluded that the challenging virus was prevented from invading petunia by the presence of TMV (Johns.).

Experiment 2. Tobacco.—Young tobacco plants were inoculated with expressed juice from tobacco infected with TMV (Johns.) as in experiment 1, and 2 weeks later were again inoculated, this time with PLV. After a further 2-week period of incubation, juice from the doubly inoculated tobacco plants was extracted and rubbed on the leaves of healthy petunia. No lesions developed on the indicator plants, again demonstrating that the presence of TMV (Johns.) had prevented invasion by the challenging PLV. In this instance the presence of one virus had prevented the invasion of a second virus in a host in which both may become systemic when introduced alone. This situation is somewhat different from that in experiment 1 in which petunia, a local lesion host for the challenging virus, is invaded systemically only by the protecting virus.

Experiment 3. Tomato.—Again, using TMV (Johns.) as the protecting virus, invasion of young tomato plants by the challenging virus PLV failed to occur during a 2-week period of incubation at 21° C.

On the basis of these experiments there is little doubt that PLV is a strain of the tobacco mosaic virus.

It should be noted, however, that in reciprocal tests using PLV as the protecting virus and TMV (Johns.) as the challenging virus, cross protection did not always occur, although it could be demonstrated in certain instances. Possibly this can be explained on the basis of the relatively slow movement of PLV resulting in its incomplete distribution at the time of invasion by the second virus. Furthermore, there was no evidence of "cross protection" when both viruses were introduced simultaneously. That is, when inoculum containing both PLV and TMV (Johns.) was applied to either healthy tobacco or tomato plants, both viruses could be recovered from a systemic locus following an appropriate period of incubation.

The Synergistic Action of PLV and TMV (Johns.) when Combined Independently with a Common Strain of PVX

The severe effects of the combined action of the petunia local lesion virus and potato virus X have already been noted in the discusssion regarding the production of the streak syndrome in tomato. In the following experiments a comparison was made of the synergistic action of PLV and TMV (Johns.) when they were combined independently with a strain of PVX. For this purpose two separate preparations of inoculum were used. (*a*) Undiluted

juice extracted from approximately one gram of fresh tobacco leaf material infected with PLV was mixed with the undiluted juice extracted from approximately one gram of leaf material from glutinosa infected with PVX. (*b*) Undiluted juice extracted from one gram of tobacco leaf material infected with TMV (Johns.) was mixed with PVX as in (*a*) above.

Both types of inoculum induced streak in tomato plants after an incubation period at 21° C in a controlled-climate chamber. With the PLV + PVX combination, streak was very severe, the plants dying within 12–15 days. In contrast, TMV (Johns.) + PVX induced a very mild streak symptom and the plants were still living at the end of the experiment, some 4 weeks after inoculation.

It is apparent, then, that both PLV and TMV (Johns.) may act synergistically with PVX to induce the streak symptom, the severity of the disease being a function of the strain of TMV involved in the virus complex.

Factors Influencing the Severity of Streak

Components of the Complex

It has been suggested above that the severity of the streak reaction in tomato is conditioned by the strain of tobacco mosaic virus present in the inoculum complex. In the following experiments the role in this regard of both the tobacco mosaic virus and the potato virus X components has been investigated.

In an effort to eliminate the gross effects which might arise from the use of crude inoculum from streak sources, only known viruses and virus mixtures were employed. Strains of TMV were first heated almost to their thermal inactivation points then inoculated to an appropriate filter plant before being accepted as inoculum for this study. Similarly, strains of PVX were also freed from possible contamination by inoculating them to glutinosa and then using only the virus which became systemic in that host.

Experiment 1.—Various combinations of the components from double-virus streak sources 34B and 18A were used as inoculum for 6-week-old tomato plants. The response of the plants following a 12-day incubation period at 21° C is indicated in Table IV. The most severe symptoms of streak

TABLE IV

A comparison of the synergistic action in tomato of various combinations of the components from double-virus streak sources 34B and 18A

Virus combination	Response of tomato host
PLV_{34B} + X_{34B}	Very severe streak
PLV_{18A} + X_{18A}	Mild streak
PLV_{34B} + X_{18A}	Mild streak
PLV_{18A} + X_{34B}	Very severe streak

resulted from inoculum containing the combination of PLV_{34B} with X_{34B} and PLV_{18A} with X_{34B}. The least severe streak developed from the combination of PLV_{34B} with X_{18A} and PLV_{18A} with X_{18A}. Assuming that the petunia local lesion virus (PLV) is uniform in virulence regardless of its source, it appears that some variation exists between the X components of the complex, X from source 18A being less severe in its synergistic action with PLV than X from source 34B. Apparently, then, the severity of the streak disease may in large part depend upon the strain of PVX participating in the complex.

Experiment 2.—Various combinations of three strains of tobacco mosaic virus, including the petunia local lesion strain, and three strains of potato virus X were used as inocula on 6-week-old tomato plants. Following an incubation period of 3 weeks at 21° C in a controlled-climate chamber, the severity of the streak symptoms was noted as an indication of the relative intensity of the interaction of the component viruses (Table V). Considerable variation was observed in the severity of the disease which developed, some combinations of viruses being very mild in their interaction while others were relatively severe.

TABLE V

A comparison of the synergistic action of three strains of tobacco mosaic virus* and three strains of potato virus X in tomato†

Virus combination	Incubation time	
	2 weeks	3 weeks
$PLV + X_{34B}$	Severe terminal necrosis	Very severe streak
$PLV + X_1$	Mild terminal necrosis	Severe streak
$PLV + X_2$	Severe terminal necrosis	Very severe streak
$TMV_{Roth.} + X_{34B}$	Necrosis of leaves and stem	Mild streak
$TMV_{Roth.} + X_1$	Mosaic mottle	Very mild streak
$TMV_{Roth.} + X_2$	Necrosis of leaves and stem	Severe streak
$TMV_{Johns.} + X_{34B}$	Necrosis of leaves	Mild streak
$TMV_{Johns.} + X_1$	Nil	Very mild streak
$TMV_{Johns.} + X_2$	Necrosis of leaves and petioles	Mild streak

*PLV = petunia local lesion virus,
$TMV_{Roth.}$ = Rothamsted strain of tobacco mosaic virus,
$TMV_{Johns.}$ = Johnson's strain of tobacco mosaic virus.
†X_1 and X_2, mild and severe strain respectively, of potato virus X from Canada Department of Agriculture, Laboratory of Plant Pathology, St. Catharines, Ontario.

Evidence from these two experiments, together with the observations made earlier in this paper, indicates that the severity of streak is a function of both virus components of the complex, a variation in the strain characteristics of either TMV or PVX being reflected in the intensity of their synergistic action in the tomato.

Incubation Temperature

The inconsistent results that were obtained during the early stages of these investigations when streak-inoculated plants were incubated under greenhouse conditions suggested that air temperature exerted an important influence

upon the expression of the streak disease. Consequently, the studies reported upon here were conducted only on plant materials maintained in controlled-climate chambers.

To determine more specifically the effect of temperature on disease expression, 6-week-old tomato plants were inoculated with infective juice from a streak source, then divided into three groups of seven each for incubation in controlled-climate chambers maintained at 16, 22, and 27° C respectively. Within 12 days all plants at the two lower incubation temperatures developed typical streak, but plants at 27° C failed to exhibit symptoms of the disease although incubated at that temperature for nearly 5 weeks. Subinoculation from these symptomless plants to tomatoes and other appropriate hosts, such as glutinosa and gomphrena, indicated that both virus components of the streak complex had become systemic during the incubation period, but had remained masked. When the symptomless tomato plants were subsequently placed at the lower incubation temperature, the masking effect was removed and the streak symptom appeared. Apparently, at the high temperature of 27° C both TMV and PVX were able to invade the tomato and become established systemically, but host sensitivity was so modified that the streak reaction did not occur.

Discussion

Streak of tomato, as it occurs in Ontario, appears, for the most part, to be the disease described by such early workers as Ainsworth *et al.* (2), Valleau and Johnson (21), and others. Certainly, the present experimental evidence indicates that the disease currently found in Ontario is practically identical with that described by Vanterpool (22), both in symptom expression and in etiology. In all instances the syndrome has been found to be double-virus in origin, the etiological complex consisting of tobacco mosaic virus and potato virus X.

Analyses of some 95 streak sources have indicated, however, that considerable variation may occur in the virus components of the streak complex. For instance, in preliminary experiments not reported here, it was found that various isolates of PVX could not readily be differentiated on the basis of symptoms induced in certain susceptible hosts. Yet, when they were individually combined with a strain of TMV, considerable differences were observed in the intensity of the action of the virus complex in the tomato. It has been concluded, therefore, that a good deal of the variation which occurs between the "severe" streak sources on the one hand, and the "mild" streak sources on the other, may be attributed to the nature of the participating strains of PVX. Indeed, further investigations comparing the physical and biological properties of potato virus X strains from streak plants would undoubtedly explain much of the erratic behavior of the streak disease in the field.

In addition to the suggestion that the severity of the streak disease may be due to variations in the virulence of the PVX component, it is also quite evident that the severe-streak so common in Ontario is mainly due to the

presence of what appears to be a unique strain or form of the tobacco mosaic virus (14). This virus (PLV), which is readily differentiated from the other common strains of TMV through the production of local lesions on the inoculated leaves of petunia and its failure to become systemic in that host, appears to be uniform in virulence regardless of its source and might well be classed as a mild-type strain when judged by its action on tomato or tobacco. However, when in combination with any one of several strains of PVX an intense synergistic interaction occurs which becomes expressed as an extremely severe streak syndrome in the tomato. It is of broad significance that most of the 95 streak samples assayed contained only this petunia local lesion strain. Of course, strains of TMV other than PLV possibly are present in the streak complex in Ontario, but the mild reaction induced by them under field conditions results in their being overlooked.

Contrary to the reports of early Canadian workers, there is no evidence at present that the streak syndrome may be induced by infection from a single virus. For example, it was claimed by Newton (16) that potato virus X alone was responsible for a streak disease of tomatoes in British Columbia. Similarly, Berkeley (4) reported upon a strain of tobacco mosaic virus which, acting alone, also produced a tomato-streak disease. Possibly these workers were dealing with isolated geographic strains of a virus which would give rise to a single-virus streak disease. In this regard, Ainsworth (1) has suggested that the major part, if not all, of the streak disease of greenhouse tomatoes in Great Britain is single-virus in origin. And yet, there remains the possibility that these workers were dealing with more than one etiological agent, possibly a combination of TMV and PVX, since there is no evidence in their reports that they had adequately eliminated all virus contaminants in their "single-virus cultures". This is not to suggest that necrotic strains of TMV may not be isolated from the tomato in Ontario; indeed, many such strains have been observed and possibly are responsible for much of the defoliation and necrosis of plants that is attributed to fungal pathogens. Nevertheless, the type of necrosis which is generally recognized as an expression of the streak disease appears to be exclusively double-virus in origin.

Probably the most outstanding factor influencing the development of streak is that of temperature. Evidence from the present investigations is that streak in tomato is either entirely masked at temperatures of 26° C and above, or the syndrome is expressed merely as a mild mosaic on the foliage. Undoubtedly, this conditioning effect of temperature explains the somewhat cyclic development of the disease in the field, appearing often during the earlier part of the season and then again later when the temperature is not at or above the critical point of 26° C for prolonged periods. Certainly, that feature whereby the virus components are maintained in the plant during the period of high temperature then act to produce a severe expression of streak as the temperature drops to a more favorable range would account for the "sudden" appearance of symptoms so frequently reported by growers who have experienced this devastating disease in their tomato crop.

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AN ANTIFUNGAL FACTOR FROM BARLEY OF POSSIBLE SIGNIFICANCE IN DISEASE RESISTANCE[1]

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Abstract

Susceptibility to *Helminthosporium sativum* in barley coleoptiles is associated with tissue senescence. Aqueous extracts of young coleoptiles show high antifungal activity. This activity disappears rapidly with age owing to the appearance of an inhibitor in the extracts. Preliminary characterization of the antifungal factor suggests an empirical formula of $C_9H_{17}O_7N$. Inhibition of activity was found to be caused by various divalent cations. The results point to the particular importance of calcium.

Introduction

The production of a toxin by the fungus *Helminthosporium sativum* and its association with disease development in barley seedlings was demonstrated by Ludwig *et al.* (4). In further studies (3), it was shown that although a loose correlation existed between toxin production and pathogenicity, other factors relating to pathogen and possibly host were also involved. The present paper represents a continuation of these studies and reports experiments on certain biochemical factors in barley that appear to be of significance in relation to *Helminthosporium* resistance and the physiology of host–parasite relations of this organism.

Tissue Senescence and *Helminthosporium* Susceptibility

The *Helminthosporium* toxin as shown previously (3) is non-specific in its action, affecting wheat, oats, barley, and even dicotyledonous species somewhat equally. It is not likely, therefore, to provide the basis of differences in varietal reaction. Its effect, as illustrated in Fig. 1, appears to be that of conditioning the barley plant to invasion by *Helminthosporium sativum*. Here, seeds were planted in gravel, and at time of emergence were watered once with the indicated concentrations of crude toxin. Spores of the organism were added to the solutions in one series and omitted in the other. It will be noted that spores alone had little, if any, effect (0 toxin). In the presence of non-lethal toxin concentrations, however, the seedlings were invaded and severely damaged (200 and 400 p.p.m. toxin). In practice, it was found difficult to reproduce the type of result illustrated in Fig. 1, except under conditions of very carefully controlled planting and environment. It was found further that the toxin could be replaced by foreign chemicals, such as salicyl aldehyde and 2,4-dinitrophenol. Environmental factors, such as crowding, and plant factors, such as physiological age, also affected the outcome. It was finally

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concluded that actively growing cells were resistant or immune and that aged or senescent cells were susceptible. The grass coleoptile is of determinate growth and senesces early in the life of the plant. It was thought, therefore, that the barley coleoptile would provide an excellent tissue on which to test the effect of age on *Helminthosporium* susceptibility.

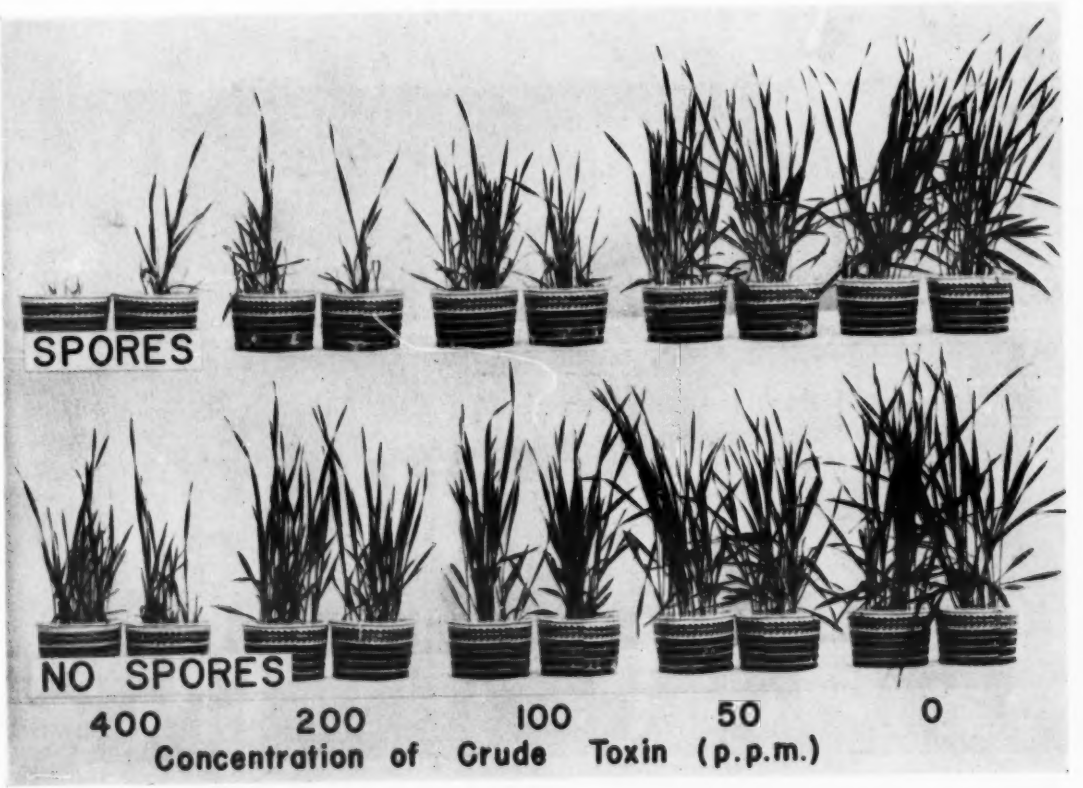
In an initial experiment, barley seedlings were grown in vermiculite, planting being done on different days in order to provide a series of coleoptiles of different ages. At the end of 2 weeks, the coleoptiles were exposed by shaking away the vermiculite, and inoculated with *Helminthosporium* spores. The vermiculite was then returned for an incubation period of 4 days before the seedlings were examined for invasion. The 5- and 6-day-old coleoptiles remained white and were apparently uninvaded. The 7- and 8-day-old coleoptiles showed typical dark *Helminthosporium* lesions around the point of inoculation. These yielded exclusively *Helminthosporium* on plating. The older coleoptiles appeared to have been invaded by a great variety of microorganisms including *Helminthosporium*.

Ludwig, White, and Unwin (5) described the growing of barley coleoptiles for use in a quick phytotoxicity test. Under the conditions employed, 5-day-old coleoptiles had a maximum growth potential as measured by elongation of an excised segment. Similarly treated segments from 6-day-old coleoptiles, however, only showed slight elongation. In the experiment illustrated in Fig. 2, 1 cm sections cut at a distance of 5 mm behind the growing point from 5- and 6-day-old coleoptiles were used. These were pretreated by soaking for 10 minutes in solutions of crude *Helminthosporium* toxin of the indicated concentrations. They were then placed on moist filter paper in petri dishes and inoculated with *Helminthosporium*. This was accomplished by preparing a water paste of spores and applying it to the mid-point of the section with a camel's hair brush. The figure shows the condition of the sections after an incubation period of 4 days. It will be noted that the 5-day-old coleoptile sections remained relatively clean in the absence of the toxin pretreatment. Invasion by *Helminthosporium* increased with increasing toxin concentration. The 6-day-old sections were susceptible without toxin treatment, but here again, damage increased with increasing treatment. An examination of the untreated 5-day-old sections under the microscope revealed that spore germination was poor and mycelial growth, scanty. This observation provides further evidence that tissue capable of active growth possesses a resistance that is lost as the cells senesce.

Biological Activity of Coleoptile Extracts

In an attempt to demonstrate an antifungal principle in the young coleoptiles, barley seedlings were grown in small flats containing vermiculite under the same environmental conditions as those previously described. Half of

FIG. 1. The effect of treatment with toxin on the susceptibility of barley seedlings to *Helminthosporium sativum*. The plants in the lower row were watered once with toxin solutions at time of emergence. Those in the upper row were treated with similar solutions to which spores had been added.



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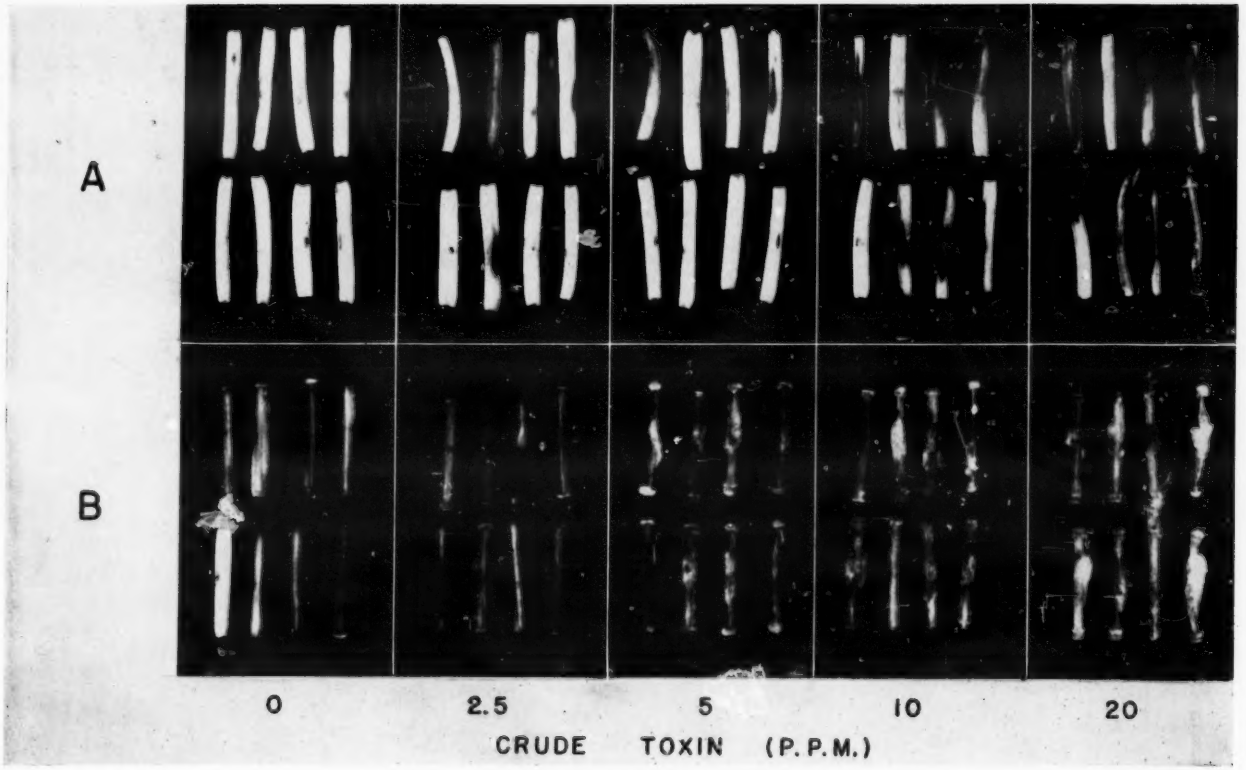

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TABLE I

Antifungal activity of extracts from 5- and 6-day-old barley coleoptiles as measured by their effect on spore germination with *Monilinia fructicola* as test organisms

Assay No.	5-day-old coleoptiles, % extract concentration						6-day-old coleoptiles, % extract concentration					
	80	40	20	10	5.0	2.5	80	40	20	10	5.0	2.5
1	100*	100	100	100	95	38	0†	0	0	0	0	0
2	100	100	100	100	96	51	0	0	0	0	0	0
3	100	100	100	100	100	41	0	0	0	0	0	0
4	100	100	100	100	91	10	0	0	0	0	0	0

*Percentage germination inhibition.
†Strong stimulation of germ tube growth occurred in this series.

the coleoptiles were harvested at the 5-day stage by pulling them from the seed. The other half were similarly harvested after 6 days. They were then homogenized in a Waring blender with 1 ml of water per gram of shoot, and boiled vigorously for approximately 5 minutes. The suspensions were filtered hot and the resultant liquor subsequently tested for antifungal activity. The standard spore drop assay using *Monilinia fructicola* as the test organism was used for this purpose. Assays were repeated on succeeding days. The results are presented in Table I. It will be noted that the extract from 5-day-old coleoptiles was highly antifungal and the solutions appeared to be relatively stable as far as activity was concerned. By 6 days, this antifungal activity had disappeared and a strong stimulation of the germ tubes was apparent in the drops. The experiments reported in the balance of this paper have to do with the antifungal factor thus demonstrated. Experiments showed that the antifungal factor could be extracted from the water with *n*-butanol, leaving an inactive aqueous residue. It was found further that the inactive liquor from 6-day-old coleoptiles, when extracted with butanol, also yielded an active fraction, as shown in Table II. In this case, recombination of the stimulatory aqueous residue and the active butanol product again resulted in stimulation. This then demonstrates that the difference between 5- and 6-day-old coleoptiles does not lie in the disappearance of the antifungal factor, but in the appearance of an inhibitor of it.

The work reported in the preceding experiments was all done with seedlings grown in complete darkness and the tissue involved was primarily that of the coleoptile although no attempt was made to remove any developing shoot tissue that was included. It was found in subsequent experiments, however, that age was not important and that inclusion of shoot tissue did not affect the outcome as long as the plants remained in good physical condition. A similar separation of activity was obtained from extracts of green seedlings.

FIG. 2. The effect of age on toxin treatment on the susceptibility of barley coleoptile sections to attack by *Helminthosporium sativum*. A, Sections of 5-day-old, and B, 6-day-old coleoptiles. The lower numbers represent concentrations of crude *Helminthosporium* toxin used as a preinoculation treatment.

TABLE II

Casamino acid as an inhibitor of the antifungal factor measured on inhibition of spore germination in *Monilinia fructicola*

Treatment	Percentage concentration					
	80	40	20	10	5	2.5
Original water extract	0*	0	0	0	0	0
Butanol phase	100	100	84	0	0	0
Aqueous phase	0	0	0	0	0	0
Butanol phase + aqueous phase	0	0	0	0	0	0
Butanol phase + 0.8% casamino acid	0	0	0	0	0	0

*Percentage inhibition.

Here, however, the chlorophyll pigments and other components in the aqueous extracts made fractionation difficult. For this reason, work was continued with etiolated seedlings.

Preliminary Studies on the Purification and Nature of the Antifungal Factor

The thermostable antifungal factor from barley shoots was separated from its inhibitor by extraction with *n*-butanol from the aqueous concentrate. However, the hydrophylic nature of the factor was indicated by the necessity of extracting six times to remove the bulk of the component from the aqueous solution. Partial purification of the butanol extract yielded a product with absorption in the ultraviolet at 260–270 mμ. This was initially of interest since the antifusarium factor in rye seedlings, 2(3)-benzoxazolinone, absorbs at 273 mμ (12) and the corn plant resistance factor, 6-methoxy-benzoxazolinone, absorbs at 288 mμ (10). The extracts still contained large amounts of yellow impurities. Subsequent attempts at purification by the use of ion exchange columns, activated charcoal, alumina, and silicic acid proved unsatisfactory, either adsorbing irreversibly or showing no preferential adsorption. However, a measure of purification by countercurrent extraction of the butanol extract with water was obtained, making use of the greater solubility of the factor in water than butanol by contrast with that of the pigmented material. Analysis of a purified fraction indicated no ultraviolet absorption, and therefore the factor is quite unlike those isolated by Virtanen (12) and Smissman and Beck (9) from rye seedlings and young corn plants, respectively.

Fractionation of the *n*-butanol extract of the factor between water and *n*-butanol in a 60-tube Craig countercurrent machine concentrated the active material in the hydrophilic end with a maximum concentration at tube 20 as shown in Fig. 3. This material exhibited 100% inhibition in the standard spore assay at a concentration as low as 25 p.p.m. An elemental analysis yielded the following: N, 5.66%; C, 42.0%; H, 6.75%. An empirical formula is $C_9H_{17}O_7N$, assuming the factor has only one nitrogen per molecule. It exhibits infrared absorption at 2225 cm^{-1}, characteristic of a nitrile. Further

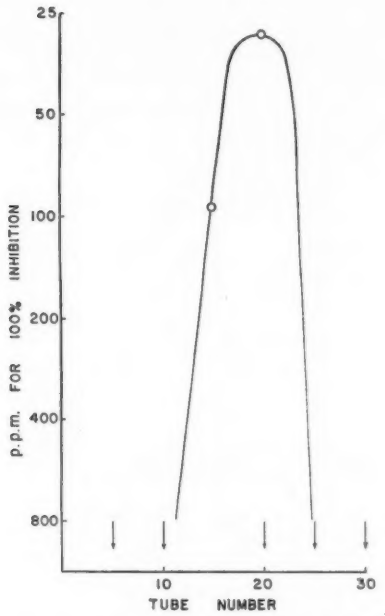

FIG. 3. Distribution of activity in tubes of a Craig liquid–liquid extractor.

confirmation is the evolution of ammonia on warming with alkali. When activity falls off, this is correlated with a loss in intensity of absorption for nitrile. A specific rotation of $[\alpha]_D^{20} + 12°$ was found. Therefore, preliminary examination of the antifungal factor from barley coleoptile would indicate a highly hydroxylated compound containing a nitrile which is labile in alkali and to a lesser extent on standing in a highly purified state.

Studies on the Nature of the Inhibitors

A series of experiments were undertaken to find what kind of substance could be used to replace the inhibitor. The first success in this regard was achieved with casamino acid as indicated in Table II. On the basis of this finding, individual and groups of amino acids were tested and found to be without effect. Finally a series of salts providing different cations and anions were examined. In these experiments the crude antifungal factor was maintained at 200 p.p.m. throughout, this concentration being approximately twice that required for 100% germination inhibition. The cation concentrations were varied. The results obtained are summarized in Table III. It will be noted here that the divalent cations are inhibitors. Calcium and magnesium appear to be outstanding in this regard, although good comparisons cannot be made since many of the salts were themselves toxic at the concentrations employed.

TABLE III

Cations and anions as inhibitors of the antifungal factor measured on inhibition of spore germination in *Monilinia fructicola*

Salt	Antifungal factor	Cation concentration, p.p.m.					
		40	20	10	5	2.5	1.25
$CaSO_4$	−	0*	0	0	0	0	0
	+	0	0	0	0	0	0
$CaCl_2$	−	0	0	0	0	0	0
	+	0	0	0	0	0	0
$Ca_3(PO_4)_2$	−	0	0	0	0	0	0
	+	0	0	0	0	0	0
$MgSO_4$	−	0	0	0	0	0	0
	+	0	0	0	0	0	61
$FeSO_4$	−	76	68	11	0	0	0
	+	88	53	28	8	0	26
$MnSO_4$	−	11	0	0	0	0	0
	+	18	8	0	0	0	0
$ZnSO_4$	−	48	44	43	29	8	7
	+	69	66	62	68	47	33
$CuSO_4$	−	100	100	100	100	98	28
	+	100	100	100	100	100	100
$CoSO_4$	−	63	63	58	32	15	0
	+	78	75	76	92	100	100
K_2SO_4	−	0	0	0	0	0	0
	+	100	100	100	100	100	100
Na_2SO_4	−	0	0	0	0	0	0
	+	100	100	100	100	100	100
NaCl	−	0	0	0	0	0	0
	+	100	100	100	100	100	100

*Percentage inhibition.

Monovalent cations and anions are without effect. Attention might be drawn here to the effect of iron, which was toxic at the higher concentrations employed. In combination with the antifungal factor a type of bimodality was obtained, inhibition at the upper end of the series being due to iron toxicity and at the lower end to the antifungal factor. At 2.5 p.p.m. the antifungal factor was completely inhibited, while at 1.25 p.p.m. there was not enough iron present to completely overcome the toxic effect of the antifungal factor. The results shown in the table suggest that the relative effectiveness of the different divalent cations varies.

In further experiments with calcium, it was found that two parts by weight of calcium were required to neutralize one part by weight of antifungal factor. This relationship, as shown in Fig. 4, held true for four test fungi. These were *Helminthosporium sativum*, *Monilinia fructicola*, *Colletotrichum atramentarium*, and *Alternaria solani*.

The possible cation relationship was further confirmed by the use of a chelating agent (ethylenediamine tetraacetic acid). The type of result obtained is illustrated in Table IV, where the results of adding various concentrations of the chelating agent to a dilution series of a non-active aqueous extract from 7-day-old coleoptiles are shown. The effect is obvious.

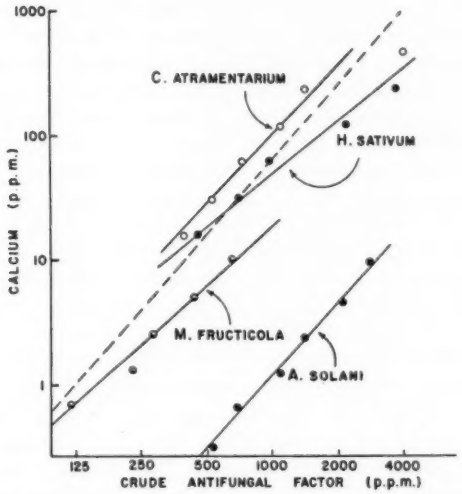

FIG. 4. The relationship between calcium and the activity of the antifungal factor from barley measured by four test fungi. The points on the curves represent ED_{50} values at different calcium levels.

TABLE IV

The effect of a chelator on "non-active" aqueous coleoptile extracts as measured by the effect on germination inhibition of spores of *Monilinia fructicola*

Chelating agent, p.p.m.	Concentration of aqueous extract, %					
	100†	50	25	12.5	6.25	3.125
100*	44	92	100	100	100	100
50	20	39	84	100	100	100
25	0	8	18	83	100	100
12.5	0	0	0	7	17	0

*The chelating agent (ethylenediamine tetraacetic acid) alone was non-toxic at this level.
†Germination was unaffected and germ tube growth stimulated by the aqueous extract.

Finally, still further evidence of the role of cations as inhibitors was obtained by drying and cooking samples of the aqueous extract. Here the inhibitor was found to remain in the ash. Preliminary analysis indicated calcium to be present in sufficient quantity to account for the observed effect.

Discussion

The nutritional theory of disease resistance, particularly when applied to an omnivorous fungus like *Helminthosporium sativum*, is far from satisfying. This organism is non-specific in its in vitro nutritional requirements, and will undergo some growth and development at very low levels of nutrition (3). Further, it produces a phytotoxin capable of killing plant cells and providing

a nutritional substratum of dead cells in which it can continue growth. An alternative explanation of resistance is a chemical one where the organism is presumably stopped by a fungicidal substance present in the plant tissue. Virtanen and colleagues (13), studying the relationship of low temperature fungi to winter survival in grasses and legumes, demonstrated the occurrence of antifungal substances as normal constituents of a number of plant species. 2(3)-Benzoxazolinone was isolated from rye and shown to occur in sufficient quantity to explain the resistance of certain varieties to *Fusarium nivale*. The corresponding factor from wheat was found to be 6-methoxy-2(3)-benzoxazolinone. Alfalfa was shown to contain an antifungal principle structurally unrelated to the benzoxazolinones although its exact nature must await the outcome of further research. Smissman *et al.* (9, 10) and Loomis *et al.* (2) found 6-methoxy-2(3)-benzoxazolinone to be associated with borer resistance in corn and extended their observations to include studies on its antifungal activity. Their findings confirm those of Virtanen *et al.* (13). Whitney and Mortimore (11) demonstrated an antifungal substance in corn stalks, and concluded that it explained differences in varietal reaction to stalk rot caused by the two fungi, *Gibberella zeae* and *Fusarium moniliforme*. Although they did not characterize the substance,* it would appear to be the 6-methoxy-2(3)-benzoxazolinone of Virtanen *et al.* (13) and Smissman *et al.* (9, 10). Ark and Thompson (1) demonstrated the occurrence of an antimicrobial substance in wheat and barley seeds. It proved to be thermostable and extractable with water and methyl alcohol. Müller (7) concluded that antifungal substances were responsible for the hypersensitive reaction of bean pods to both *Phytophthora infestans* and *Monilinia fructicola*. He concluded further that these substances were not normally present in the bean tissue, but were produced in response to infection by the organisms. Millerd (6) arrived at similar conclusions with respect to mildew resistance in barley.

The antifungal factor from barley described in this paper is distinct from the benzoxazolinones. The fact that it may be neutralized in aqueous plant extracts by a naturally occurring inhibitor (inhibitors) is a new observation. This is of particular interest since it is the appearance of the inhibitor rather than the disappearance of the antifungal factor which leads to inactivity in extracts of older tissue. Further, the association of both activity in the aqueous extracts and the reaction to *Helminthosporium sativum* with age of tissue suggests that the over-all phenomenon is related to resistance and susceptibility.

Divalent cations were found to be the inhibitors in the aqueous extracts with evidence pointing to the particular importance of calcium. Much remains to be learned about the role of this element in plant growth and nutrition (8). The results obtained here could be explained on the basis of its absence from the young tissue in ionic form or changes in permeability permitting its diffusion from the cell to infection points.

*NOTE ADDED IN PROOF: Whitney and Mortimore have since identified the antifungal substance from corn as 6-methoxybenzoxazolinone as reported in a letter submitted to Nature.

The activity of the antifungal factor and its relationship to calcium is non-specific since it affects the four test organisms employed equally. There is a definite molar relationship between the antifungal factor and the amount of calcium required to neutralize it as demonstrated in Fig. 4. The nature of this relationship, however, is as yet undetermined.

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THE ESTIMATION OF NUCLEIC ACIDS IN SOME ALGAE AND HIGHER PLANTS[1]

ROBERT M. SMILLIE[2] AND G. KROTKOV

Abstract

Several current methods for the extraction and estimation of nucleic acids in biological materials were applied to *Euglena* and other plants. The efficiency of both the preliminary extractions for acid-soluble-P and lipid-P and the subsequent extraction of the nucleic acids was studied. A relatively high acid concentration (15% TCA) was required to directly extract all the acid-soluble phosphates. These conditions appeared to remove a small amount of the RNA. Lower acid concentrations as used in the Ogur–Rosen method (2% PCA) failed to extract all the acid-soluble phosphates. By using a modification of the Ogur–Rosen initial extraction method, the acid-soluble phosphates were quantitatively extracted without loss of RNA. After removal of the acid-soluble phosphates and lipid phosphates, the plant nucleic acids were quantitatively extracted by either the Schmidt–Thannhauser or Schneider methods. In many of the plants tested, the presence of pentose-containing polysaccharides, protein degradation products, or polyphosphate (algae only) interfered in estimations based on either the Schneider or Schmidt–Thannhauser procedures. Such interfering substances in the Schmidt–Thannhauser method were eliminated by the use of an anion exchange resin. Details are given of a modified Schmidt–Thannhauser procedure which should be suitable for a wide range of plants. The modified procedure may be simplified for *Euglena* and some higher plant tissues depending on the nature and quantities of interfering substances present. Methods are also given for the quantitative separation of plant RNA nucleotides by paper chromatography and by ion exchange paper chromatography.

Introduction

The role of nucleic acids in cellular growth and metabolism forms an increasingly important field of study in botanical research. Adequate methods for the extraction and estimation of DNA[3] and RNA from both the higher plants and algae are necessary in order to facilitate such studies. Most methods used for the extraction and estimation of nucleic acids from biological materials are based on procedures which were developed by Schneider (28) and Schmidt and Thannhauser (27). Although both of these procedures were developed specifically for animal tissues, they have been applied directly, or in modified forms, to a wide range of organisms. Plants have presented a special problem because of the larger amounts of interfering substances which they frequently contain. A detailed investigation of the estimation of nucleic acids in plants was first undertaken by Ogur and Rosen (24). They developed a method based on the differential extraction of RNA and DNA by PCA at different temperatures. The Ogur–Rosen procedure has since been adopted for the estimation of nucleic acids in a number of studies on plant tissues. Unfortunately, in many of these cases, no attempt was made to first test the suitability of the method for the tissue involved.

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Contribution from the Department of Biology, Queen's University, Kingston, Ontario. This investigation was supported by the National Research Council of Canada.
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[3]Abbreviations used in this paper: DNA for deoxyribonucleic acid; RNA for ribonucleic acid; PCA for perchloric acid; TCA for trichloroacetic acid.

There is ample evidence that the Ogur–Rosen procedure, although satisfactory for some tissues, may not be so for others (2, 7, 16, 21, 23, 26). The same is probably true for the Schneider and Schmidt–Thannhauser procedures, when they are used for the estimation of nucleic acids in plants.

In this paper, a comparison is made of the Schneider, Schmidt–Thannhauser, and Ogur–Rosen procedures for the estimation of the nucleic acids in a number of algae and higher plants. It is noted where errors in the estimations are likely to occur and modifications to avoid these are suggested. Details are presented of a modified Schmidt–Thannhauser procedure, which should have a wide application to nucleic acid estimations in plant tissues. The quantitative extraction and separation of the RNA nucleotides are also discussed.

Materials and Methods

Euglena gracilis,[4] strain Z, was grown heterotrophically (11) in the light or in the dark. *Chlorella vulgaris*[4] was grown in light on the inorganic medium of Emerson and Lewis (8) modified to include ethylenediamine tetraacetate. The gas phase was 5% CO_2 in air. *Navicula pelliculosa*[4] was cultivated in aerated inorganic medium (17) and illuminated by white light. All cells were harvested during their log phase of growth. Pea (*Pisum sativum* var. Laxton's Progress), beet (*Beta vulgaris*), and barley (*Hordeum vulgare*) plants were grown in flats in soil under greenhouse conditions.

Total phosphate was estimated following the procedure of Allen (1). Ribose was estimated by a modification of the orcinol reaction (19). Ribose (10 μg) was used as a standard. Burton's (4) modification of the diphenylamine reaction was used to estimate deoxyribose. Hydrolyzed DNA (4) (National Biochemicals Corporation's highly polymerized DNA) containing 6.8 μg P was used as a standard. These modifications of the orcinol and diphenylamine reactions are both more sensitive and are subject to less interference than the original procedures (4, 19).

Results

Extraction of the Acid-Soluble-P and Lipid-P

Since most methods for the estimation of nucleic acids depend on pentose, phosphate, or ultraviolet light absorption measurements, nucleotides and other substances which would interfere in these measurements should be eliminated. The extraction of tissues with cold acid removes many of these substances (24, 27, 28). Lipid phosphates may be removed from the tissues by a series of extractions with organic solvents. The initial acid extractions should not remove or degrade nucleic acids. RNA is slowly extracted by dilute acid (24), while DNA may be modified in such a way as to cause difficulties in its subsequent separation from RNA by the Schmidt–Thannhauser method (34). By first establishing suitable initial extraction procedures

[4]We are indebted to Dr. S. H. Hutner, Haskins Laboratory, New York, for a culture of *Euglena gracilis*, to Dr. P. Gorham, National Research Council, Ottawa, for a culture of *Chlorella vulgaris*, and to Dr. R. C. Starr, University of Indiana for the *Navicula pelliculosa*.

for the quantitative extraction of acid-soluble and lipid phosphates, many of the problems that are frequently encountered in the subsequent extraction and estimation of the nucleic acids are avoided.

In the Schneider and Schmidt-Thannhauser procedures acid-soluble substances are usually removed by direct extraction of the tissues with 7 to 15% TCA (6, 27, 28). The Ogur–Rosen method (24) employs 2% PCA after a series of extractions with organic solvents. Direct extraction of *Euglena* cells by three concentrations of acids are shown in Table I. The amount of the acid-soluble-P extracted depended on the acid concentration of the extractant, while the lipid-P extracted remained practically the same.

TABLE I

Extraction of acid-soluble-P from *Euglena* by different concentrations of acids

Extracting acid for acid-soluble-P	Total μg P extracted		
	Acid-soluble-P	Lipid-P	Total-P
15% TCA	166	82	248
10% PCA	145	87	232
2% PCA	82	88	170

NOTE: Samples of *Euglena* cells containing the same dry weight of cells were extracted for 30 minutes at 0–4° C with the different concentrations of acids. The cells were centrifuged, washed with the same concentration of the acid, and then extracted for lipid-P according to the Schmidt–Thannhauser method (27).

A similar series of extractions are shown in Fig. 1, except that the Ogur–Rosen preliminary extraction procedure was followed for the 2% PCA extraction. Following the initial extractions, the RNA and DNA were extracted and separated by the Schmidt–Thannhauser procedure and the phosphate contents of the two fractions were determined. The combined acid-soluble-P and lipid-P levels again depended on the concentration of the extracting acid. The P content of the RNA fraction showed an opposite trend. The total-P extracted by the three procedures was approximately the same.

In Fig. 2 the phosphate analyses for the RNA fractions (Fig. 1) are compared with values obtained on the same fractions by pentose and ultraviolet light absorption measurements. Only in the case of the extraction with 15% TCA did all three estimations agree. These values were similar to the other pentose and ultraviolet light absorption results, although the values obtained after the 2% PCA extraction were about 5% higher than those for 15% TCA and 10% PCA initial extractions. Hence in the procedures where 10% PCA and 2% PCA were used, the RNA fraction was contaminated by some non-nucleotide-P, while where 15% TCA was employed, the RNA fraction was free of this non-nucleotide-P. It is unlikely that the RNA fraction after the 15% TCA extraction contained some residual acid-soluble nucleotide-P, since the lower acid concentrations resulted in incomplete extraction of only non-nucleotide-P. Assuming that no RNA was extracted during the Ogur–Rosen initial extraction procedure with 2% PCA (there is no indication in the literature that RNA would be appreciably extracted under these mild conditions), then about 5% RNA was lost with the higher concentrations of

extracting acid. Alternatively, the extra 5% nucleotide-P after the 2% PCA extraction may have been unextracted acid-soluble nucleotide-P. The experiments which follow indicated that the higher acid concentrations (10% PCA and 15% TCA) did in fact remove a small amount of the RNA.

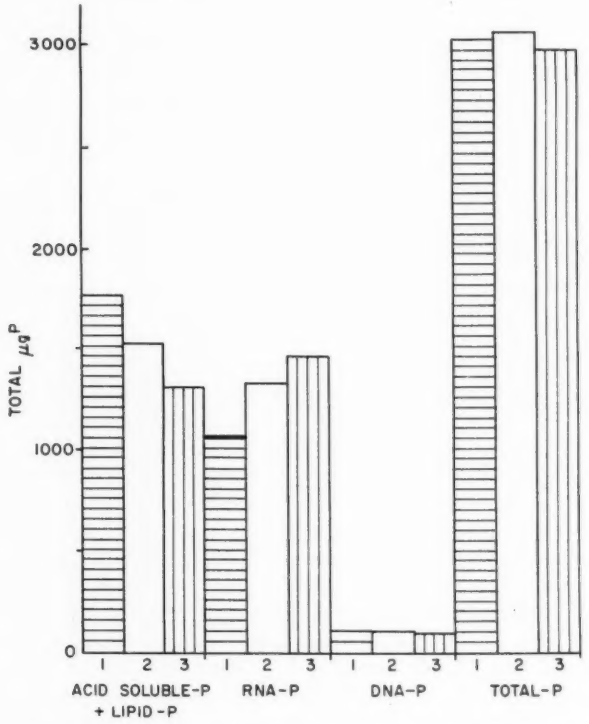

FIG. 1. Phosphate fractions extracted from *Euglena* by different methods. In experiments 1 and 2, the cells were extracted directly with 15% TCA and 10% PCA respectively. Lipid phosphates were then extracted with ethanol:ether mixtures (24). In experiment 3, the acid-soluble and lipid phosphates were extracted by the Ogur–Rosen method (24). In all three experiments after the initial extractions, the nucleic acids were separated by the Schmidt–Thannhauser procedure (27). The total-P content of the individual fractions was determined.

From the experiments presented above it was apparent that neither the usual Schmidt–Thannhauser–Schneider nor the Ogur–Rosen preliminary extraction technique was entirely adequate for *Euglena*. The former method extracted all the acid-soluble-P and lipid-P, provided high enough acid concentrations were used (or a longer time for extraction was allowed), but at the same time it appeared to degrade a small part of the RNA. The latter method incompletely extracted the acid-soluble-P.

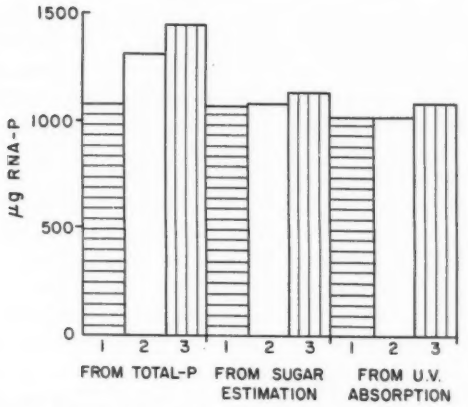

FIG. 2. RNA content of *Euglena* after preliminary extraction of acid-soluble and lipid phosphates by three different methods. Experimental procedure as in Fig. 1.

A modification of the Ogur–Rosen preliminary extraction procedure was next tried.[5] *Euglena* cells were extracted with methanol at 4° C and re-extracted with methanol containing 0.05 *M* formic acid. This was followed by extraction at 4° C with 5% TCA. The residual lipid-P was extracted with alcohol:ether mixtures (24). Details of the extraction procedure are given in a later section and Fig. 3. All individual extractions were done at least twice and the extracts pooled. In Table II this method of extraction is compared with the Ogur–Rosen preliminary extraction procedure. In both cases the nucleic acids were finally extracted by hot 5% PCA (28).

TABLE II

A comparison of two procedures* for the extraction of acid-soluble-P and lipid-P from *Euglena*

	μg P/mg *Euglena* dry wt.	
	Method 1	Method 2
1st extraction	1.49	1.18
2nd extraction	0.95	0.53
3rd extraction	0.29	0.58
Total acid-soluble-P and lipid-P	2.73	2.29
Total nucleic acid-P (from total-P)	2.00	2.46
Nucleic acid-P from sugar analyses:		
RNA-P	1.84	1.83
DNA-P	0.180	0.176
RNA-P + DNA-P	2.02	2.01

*Method 1. (Methanol – 5% TCA): 1st extraction, methanol followed by methanol containing 0.05 *M* formic acid; 2nd extraction, 5% TCA; 3rd extraction, ethanol:ether mixtures.
Method 2. (Ogur–Rosen): 1st extraction, 70% ethanol followed by 70% ethanol containing 0.1% PCA; 2nd extraction, ethanol:ether mixtures; 3rd extraction, 2% PCA. In both methods the nucleic acids were subsequently extracted with hot 5% PCA (28).

[5]The authors are indebted to Dr. H. Stern, Department of Agriculture, Ottawa, for details of this procedure.

The values for total nucleic acid-P, based on either sugar or total phosphate determinations, agreed when the methanol – 5% TCA preliminary extraction procedure was used. These values also agreed with the nucleic acid-P based on sugar estimations after the Ogur–Rosen preliminary extraction procedure, but not with the total-P analyses of the nucleic acid fraction. Thus it appeared that the methanol – 5% TCA extraction procedure quantitatively extracted non-nucleic acid-P without loss of RNA. Identical results were obtained if acetone was substituted for methanol, or 5% PCA for TCA.

The data of Figs. 1 and 2 show that it was necessary to use an acid concentration as high as 15% TCA with the Schmidt–Thannhauser initial extraction procedure before all the non-nucleic acid-P was extracted, whereas the methanol – 5% TCA preliminary extraction procedure removed all the acid-soluble-P (Table II). The reason for this is apparent from the data of Table III where the efficiency of a 5% TCA extraction before and after treatment with methanol is compared. The acid-soluble-P was removed slowly by

TABLE III

Extraction of phosphate-containing compounds from *Euglena* cells with and without preliminary treatment with methanol

Fraction	Extraction solvent	Volume, ml	μg P	Total μg P of fraction
Method 1:				
Acid-soluble-P	5% TCA (0–4° C)	10	33	
		10	39	
		10	28	100
Lipid-P	Ethanol (boiling)	15	188	
	Ethanol:ether (3:1) (boiling)	10	7	
	Ether (boiling)	5	1	196
Nucleic acid-P	5% PCA (90° C)	10	316	
		10	10	326
Residue	Digest with 70% PCA (180° C)		7	7
			Total-P	629
Method 2:				
Methanol-soluble-P	Methanol (0–4° C)	10	181	
	Methanol – formic acid (0–4° C)	10	12	
	Methanol – formic acid (0–4° C)	10	3	196
Acid-soluble-P	5% TCA (0–4° C)	10	112	
		10	15	
		10	5	132
Lipid-P	Ethanol (boiling)	15	23	
	Ethanol:ether (3:1) (boiling)	10	5	
	Ether (boiling)	5	2	30
Nucleic acid-P	5% PCA (90° C)	10	241	
		10	9	250
Residue	Digest with 70% PCA (180° C)		6	6
			Total-P	614

NOTE: Samples of *Euglena* cells (100–120 mg dry wt.) were extracted as outlined below. The total-P content of each extract and of the final residue was determined. In method 1 the cells were extracted directly with cold 5% TCA. In method 2 the acid extraction was carried out after the cells had been extracted with methanol.

5% TCA from fresh cells, but after treatment with methanol the extraction was rapid. Following the initial extractions, the total nucleic acid was extracted with hot 5% PCA (28) and the total-P content of the extract was determined. Incomplete extraction of the acid-soluble-P by 5% TCA without prior treatment of the cells with methanol was reflected in the higher total-P values for the nucleic acid fraction.

Other experiments have shown that if *Chlorella* or *Navicula* cells, or homogenates of pea leaves or beet roots (in water or methanol), were extracted first with cold methanol, a rapid and quantitative extraction of the acid-soluble-P took place upon the addition of cold 5% TCA or PCA. There was no evidence of degradation and extraction of the nucleic acids during the short time required for the acid extractions.

The initial methanol extraction removed some substances which were normally extracted with the acid-soluble-P fraction (i.e., direct extraction of tissues with cold, dilute acid). In some experiments it may be required that the acid-soluble-P fraction be retained for additional analyses, and in such cases the methanol extraction may be avoided as follows. A suspension of cells or tissue homogenate in water or 5% TCA is mixed with nine volumes of cold ether and shaken vigorously for 1.5 minutes. The mixture is rapidly centrifuged and the ether layer is removed by suction. The aqueous layer is then extracted with 5% TCA. The ether treatment has a similar effect to an initial extraction with methanol in that the acid-soluble phosphates are now readily extractable. The total-P removed in the ether layer is almost negligible.

Extraction of the Nucleic Acids from Euglena

Reports in the literature indicate that of the three common methods used to extract nucleic acids, hydrolysis and extraction of RNA by alkali (Schmidt–Thannhauser) or extraction by hot 5% PCA (Schneider), are quantitative, whereas differential extraction and separation of RNA and DNA by PCA (24) is not always so. Therefore in the experiments described above, either the Schmidt–Thannhauser or Schneider procedures were employed for the extraction of the nucleic acids. Either method was found to be satisfactory for *Euglena*. For routine estimations, the method of Schneider was preferred because it could be carried out more rapidly. The color given by purified DNA in the orcinol reaction was only 0.3% of that given by RNA (on a molar basis) at 670 mμ. Thus it was unnecessary to correct for the presence of DNA when RNA was estimated in the Schneider method by its pentose content. RNA did not form any color in the diphenylamine reaction for DNA.

If *Euglena* cells were grown in the dark, or if older, light-grown cultures were used, non-nucleic acid-P (polyphosphate) was found in the nucleic acid fraction (30) and this invalidated the analyses for RNA based on total phosphate. The sugar and ultraviolet light absorption analyses were used to estimate quantitatively the DNA and RNA present.

Several methods for the extraction of nucleic acids from *Euglena* are compared in Table IV. The cells were initially extracted to remove acid-soluble-P and lipid-P and they were then divided into several portions for the extraction of their nucleic acids. Methods 1 and 2, viz. the Schneider and Schmidt–Thannhauser methods, gave essentially the same values for RNA and DNA. In the latter, KOH quantitatively solubilized both RNA and DNA. On acidification of the hydrolyzate, the DNA was almost completely precipitated, while the RNA nucleotides remained in solution. Methods 3 and 4 were the Schneider and Schmidt–Thannhauser procedures used on the extracts of nucleic acids with hot 10% NaCl (6). An anomaly occurred in method 4, in which incomplete precipitation of the DNA was encountered upon acidification of the KOH digest. This difficulty was eventually surmounted by the addition of Mg^{++} ions (10^{-3} *M*) after the acidification step as suggested by Markham (19). The precipitation of DNA was then quantitative as indicated by deoxyribose analyses (4). The Ogur–Rosen procedure when applied to *Euglena* incompletely extracted the RNA. Two other methods,

TABLE IV

Estimation of RNA and DNA in *Euglena* by several methods

Method	Procedure	Total μg P	
		DNA-P	RNA-P
1. Schneider (ref. 28)	5% PCA extract (90° C, 15 min)	27.9	320
2. Schmidt–Thannhauser (ref. 27)	KOH digestion, followed by acid precipitation of DNA		
	RNA fraction	0.9	326
	DNA fraction	27.0	0
		27.9	326
3. NaCl extraction + Schneider	Extract first with 10% NaCl (100° C, 1.5 hr) and precipitate the nucleic acids with ethanol (2.5 volumes)		
	5% PCA extract of nucleic acid precipitate	25.6	306
	5% PCA extract of NaCl extraction residue	2.0	25
		27.6	331
4. NaCl extraction + Schmidt–Thannhauser	As in 3, only nucleic acid precipitate digested with KOH as in 2		
	RNA fraction	18.1	289
	DNA fraction	6.8	0
	5% PCA extract of NaCl extraction residue	2.2	23
		27.1	312
5. Ogur and Rosen (ref. 24)	Extract with cold 1 *N* PCA for RNA and 0.5 *N* PCA (70° C, 20 min) for DNA		
	RNA fraction	0	189
	DNA fraction	26.5	131
		26.5	320

NOTE: The starting material was *Euglena* cells which had been extracted for acid-soluble-P and lipid-P by the methanol – 5% TCA extraction method as given in Table II.

those of Chiba and Sugahara (5) and Kirby (13), which have been used to separate RNA and DNA in plant and animal tissues respectively, have also been tried. They did not separate the two nucleic acids from *Euglena*.

Extraction of Nucleic Acids in Plants Other than Euglena

After the removal of the acid-soluble-P and lipid-P by the methanol – 5% TCA extraction procedure, it was still not always possible to directly apply either the Schneider or the Schmidt–Thannhauser methods to the subsequent extraction and estimation of the nucleic acids. In *Euglena* grown under certain conditions and in most other algae, the occurrence of polyphosphate invalidated the total-P values. Since polyphosphate has not been found in higher plants (12), the total-P of the nucleic acid fractions probably gives a reliable value for their nucleic acids. An approximate estimate of the total nucleic acid may also be obtained from ultraviolet light absorption measurements. However, the exact extinction coefficients of most plant nucleic acids are not known and protein degradation products may contribute to the ultraviolet light absorption of the extracts (22). The presence of pentose-containing polysaccharides, which were not removed by the initial acid and organic solvent extractions and which thus rendered RNA values based on ribose determinations too high, were encountered in several plants. The inadequacy of either the Schmidt–Thannhauser or the Schneider methods for the determination of RNA from *Chlorella*, which contains both polyphosphate and non-nucleic acid pentose, is demonstrated by the data in Table V. Both the ribose and total-P values were considerably in excess of those obtained from ultraviolet light absorption measurements. In the Schmidt–Thannhauser procedure it was observed that about half of the total pentose was insoluble in KOH and was removed by centrifugation of the KOH digest. However, the pentose-RNA value obtained was still too high when compared with the ultraviolet light absorption values, which indicated that some non-nucleic acid pentose was still present in the RNA fraction. Polyphosphate is labile in alkali (14) and also contaminated the RNA fraction.

TABLE V

Estimation of *Chlorella* nucleic acid by the Schneider and Schmidt–Thannhauser procedures

Method	Total μg P		
	Total nucleic acid-P		RNA-P from pentose
	From U.V.	From total-P	
1. Schneider 5% PCA, 90° C, 15 min	151	335	519
2 Schmidt–Thannhauser KOH digestion (total for all fractions)	163	338	434
Individual fractions:			
(*a*) Insoluble in KOH	4	1	211
(*b*) RNA fraction	142	306	217
(*c*) DNA fraction	17	31	6

NOTE: The starting material was *Chlorella* cells which had been previously extracted for acid-soluble-P and lipid-P by the methanol – 5% TCA extraction method as given in Table II.

Two modifications were investigated in an effort to circumvent the interference of non-nucleic acid pentose and phosphate in RNA assays in plants: first, an additional purification step involving the extraction of nucleic acids with 10% NaCl; and second, the separation of the RNA nucleotides from the non-nucleotide phosphate and pentose by ion exchange chromatography.

The Extraction of Nucleic Acids with NaCl

The extraction of nucleic acids by NaCl solutions was employed by Davidson *et al.* (6) for animal tissues as an additional purification step during the extraction of tissue nucleic acids by the Schmidt–Thannhauser method. Martin and Morton (21), Stern (personal communication), Thomas and Sherratt (32), and Williams (35) have applied procedures utilizing NaCl solution for the extraction of nucleic acids from plants. From Table IV it can be seen that 10% NaCl at 100° C extracted approximately 90% of the total nucleic acid from *Euglena*. The remaining 10% nucleic proved difficult to extract and in one experiment after five successive 1-hour extractions, 8.3% was still unextracted. Similar experiments with *Chlorella* were disappointing. The results of three experiments are shown in Table VI. The *Chlorella* cells were initially extracted for acid-soluble-P and lipid-P and then were extracted with the hot 10% NaCl. The best result (experiment 3) indicated that 50% of the total nucleic acid was extracted but only 16% of the DNA. NaCl

TABLE VI

Extraction of nucleic acids from *Chlorella* with 10% NaCl

Experiment 1.—*Chlorella* cells were harvested from a rapidly growing culture and were extracted for acid-soluble-P and lipid-P by the methanol – 5% TCA extraction procedure as given in Table II. Fifteen milligrams of extracted *Chlorella* powder were extracted twice with 8 ml of 10% NaCl containing 0.016 *M* tris buffer, pH 7.1, for 30 min at 100° C, with continuous stirring. Nucleic acids in the NaCl extract were precipitated with 2.5 volumes of absolute ethanol. The optical density at 260 mμ ($O.D._{260}$) of the various extracts was taken as an approximate index of their nucleic acid contents.

	% total $O.D._{260}$
Hot 5% PCA extract of NaCl residue	79
Hot 5% PCA extract of ethanol precipitate	21

Experiment 2.—As above, only cells were obtained from an older culture. The estimations were made directly on the NaCl extract.

	% total $O.D._{260}$	% total DNA*
NaCl extract	45.5	11.4
NaCl residue	54.5	88.6

Experiment 3.—As in experiment 2.

	% total $O.D._{260}$	% total DNA*
NaCl extract	50.5	16.4
NaCl residue	49.5	83.6

*DNA estimated by the diphenylamine reaction (4).

extractions were not carried out on other plants. The experiments described, however, show that before NaCl extraction procedures can be used in conjunction with the quantitative extraction and estimation of nucleic acids from a particular plant tissue, the efficiency of the NaCl solution extraction must be established.

The Use of Ion Exchange Chromatography in the Estimation of Nucleic Acids in Plants

The use of an anion exchange resin as a means of eliminating interfering substances in the estimation of nucleic acids from plants such as *Chlorella* was investigated. *Chlorella* cells were extracted by the methanol – 5% TCA procedure described previously and the nucleic acids were further separated and extracted by the Schmidt–Thannhauser method. The neutralized RNA fractions were poured through a 5 cm × 0.5 sq. cm column of Dowex-1 anion exchange resin, chloride form, 200 mesh. Elution was carried out with HCl – NaCl solution (20 ml of 10 *N* HCl + 5.6 g NaCl in 240 ml of water) after the column was first washed with 0.01 *M* NaCl. Table VII shows the optical density of successive 5-ml portions of the eluate. Most of the ultraviolet light absorbing compounds were eluted in the first 15 ml. Table VIII shows the results of assays on samples of KOH digests of *Chlorella* cells and pea leaves before absorption on and after elution from a Dowex-1 column. For *Chlorella*, the initial phosphate and pentose values of the KOH digest containing RNA were much too high. The values obtained by the three different assays on the eluate were almost identical and demonstrated that contaminating non-nucleotide-P and pentose had been removed.

TABLE VII

Recovery of *Chlorella* RNA nucleotides from a Dowex-1 resin column

Fraction (each 5 ml)	O.D.$_{260}$ of fraction	Fraction (each 5 ml)	O.D.$_{260}$ of fraction
1	2.744	5	0.078
2	4.344	6	0.017
3	1.594	7	0.002
4	0.304	8	0.000
			9.083

NOTE: Total O.D.$_{260}$ of fractions = 45.4,
Total O.D.$_{260}$ of the original KOH digest put through column = 46.1,
% recovery = 98.

Similar results were found for pea leaves (Table VIII), except that in addition to the values calculated from the ultraviolet light measurements, the total-P values before and after the ion exchange chromatography closely agreed. This correlation pointed to the absence of contaminating non-nucleotide-P in the KOH digest. Hence it was not necessary to use the column procedure with estimations on pea leaves, since direct estimations of total-P sufficed. The same comments applied to estimations on beet root. Some RNA and DNA contents of pea leaves and beet roots are shown in Table IX.

Preliminary experiments indicated that absorption and subsequent elution of KOH extracted cells of the diatom *Navicula pelliculosa* quantitatively yielded RNA nucleotides which were free of substances interfering in total phosphate and ribose estimations. Like *Chlorella*, KOH digests of *Navicula* cells (previously extracted for acid-soluble-P and lipid-P) contained non-nucleotide-P and pentose.

Recently, Deken-Grenson and Deken (7) have suggested the use of an ion exchange resin to eliminate substances which interfere in the orcinol reaction after the extraction of RNA by the Schmidt–Thannhauser procedure.

TABLE VIII

The removal of non-nucleic acid-P and pentose from RNA hydrolyzates by the absorption of RNA nucleotides on Dowex-1 and elution with HCl–NaCl solution

Assay based on:	Total μg P	
	KOH digest of tissue RNA	Eluate from Dowex-1
A. RNA from *Chlorella*		
Total phosphate	528	152
Pentose	555	155
U.V.	161	154
B. RNA from *Pisum sativum* leaves		
Total phosphate	197	194
Pentose	293	203
U.V.	217	215

NOTE: *Chlorella* cells or pea leaves were extracted for acid-soluble-P and lipid-P by the methanol – 5% TCA initial extraction method as given in Table II. RNA was then extracted from this material and separated from DNA by the Schmidt–Thannhauser method (27). The RNA nucleotides were absorbed on Dowex-1 and eluted together as described in the text.

TABLE IX

Nucleic acid contents of pea leaf and beet root

	μg P/g fresh weight	
	RNA	DNA
Pea leaf		
Young	710	31.0
Mature	140	10.4
Beet root	24.6	2.41

Details of Procedures for the Extraction, Separation, and Estimation of Nucleic Acids in Plants

Details of a procedure, based on the experiments described above, for the extraction, separation, and estimation of RNA and DNA in plant tissues is described below and summarized in Fig. 3. The method is in effect a modified Schmidt–Thannhauser procedure. It may be simplified depending on the properties of the plant tissue (see below).

A sample of cells or tissue (20–100 mg dry weight) is extracted with 10 ml of methanol and is re-extracted at least twice with 10 ml of methanol or methanol containing 0.05 *M* formic acid. The method of extraction is as follows. A fine suspension of the plant material in the extracting solvent, which is contained in a centrifuge tube, is stirred for 20 seconds with a glass rod. The suspension is allowed to stand for 2 minutes, followed by a further 20 seconds of stirring. The suspension is then centrifuged. If at any time lumps are present in the residues, the latter are homogenized in the tubes

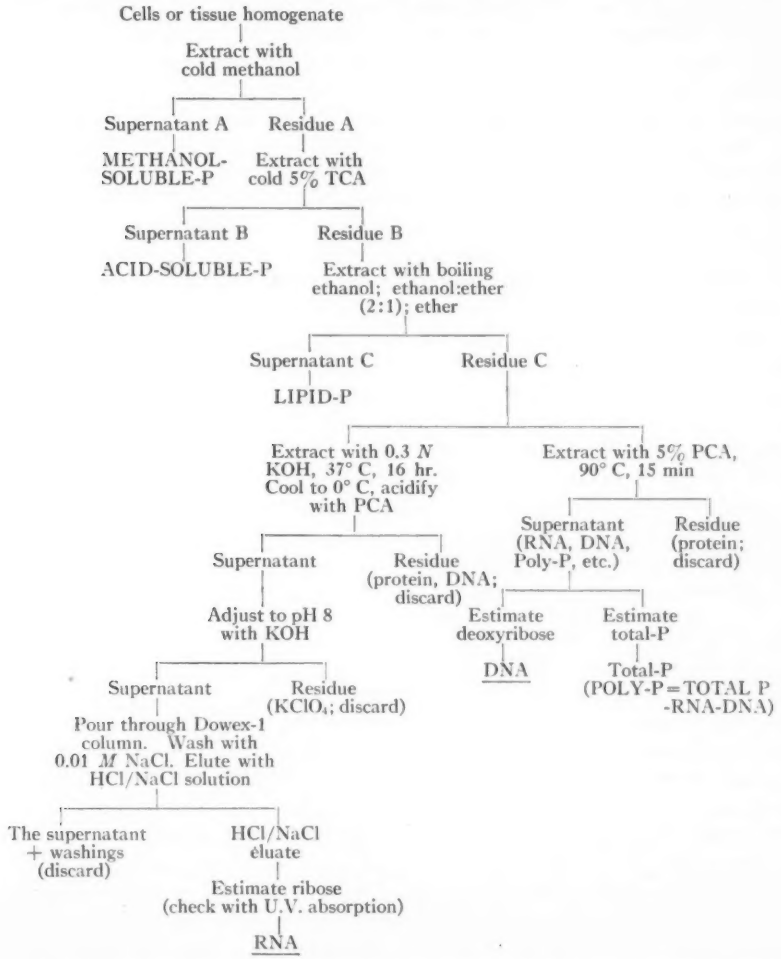

FIG. 3. General extraction procedure for the estimation of nucleic acids in plants.

with closely fitting mechanically driven glass pestles until a fine suspension is obtained. The residue is then extracted in the same centrifuge tubes with 2×10 ml of 5% TCA at 4° C and then washed once with 10 ml of cold 95% ethanol. Ten-milliliter lots of the following are then used for further extraction of the residue—95% ethanol (twice), ethanol:ether (2:1), and ether. For each extraction the solvent is allowed to boil for 20 seconds. The insoluble residue is then carefully dried and powdered. For RNA the dried residue is extracted at 37° C with 0.3 *N* KOH for 16 hours. The digest is cooled in ice water, $MgCl_2$ is added to 10^{-3} *M*, and the pH is adjusted to approximately 2 with PCA. One volume of 95% ethanol is added and the mixture is allowed to stand at 0° C for 20 minutes. It is then centrifuged and the precipitate is washed with ice-cold 1% PCA. The supernatant and washings are adjusted to pH 8 with KOH, chilled, centrifuged, and the precipitate washed with small volumes of cold water. The combined supernatants are run through a 5 cm × 0.5 sq. cm column of Dowex-1 resin, chloride form, 200 mesh. The column is washed with 0.01 *M* NaCl. The nucleotides are retained on the column and are eluted together with HCl–NaCl solution (20 ml HCl + 5.6 g NaCl in 240 ml of water). Twenty-five milliliters of eluate are collected. The ribose content of the eluate gives an estimate of the RNA. This can be checked by absorption readings at 260 mμ.

DNA can be estimated by extraction of the original dried residue (residue C, Fig. 3) with 5 ml of 5% PCA at 90° C for 15 minutes. After centrifugation the precipitate is well washed with 5% PCA at 90° C. The DNA in the combined extracts is assayed for its deoxyribose content by the diphenylamine reaction (4). The standard is purified DNA hydrolyzed under the same conditions. Extraction of residue C with 5% PCA rather than the DNA-protein precipitate after the KOH digestion is preferred in case the precipitation of the DNA in the latter is not quantitative. Further, if it is desired to know the polyphosphate content (of algae), the total-P of the PCA extract of residue C is also determined and the polyphosphate calculated by the difference between this and the total nucleic acid values.

Modified Procedures for Certain Plants

The procedure given above is probably applicable to most plants. For some plants, however, the procedure outlined in Fig. 3 can be simplified. Thus for *Euglena* and the higher plant tissues tested, the absence of certain interfering substances rendered the ion exchange treatment unnecessary. Further, it was not necessary to separate RNA from DNA and a simple extraction of the nucleic acids with 5% PCA sufficed. A simplified procedure for *Euglena* is shown in Fig. 4. Figure 5 shows the procedure modified for the extraction and estimation of the nucleic acids in pea leaf and beet root. The latter procedure is probably satisfactory for a wide range of higher plant tissues. However, before such a simplified procedure is applied to other higher plants, the presence or absence of interfering substances should be established.

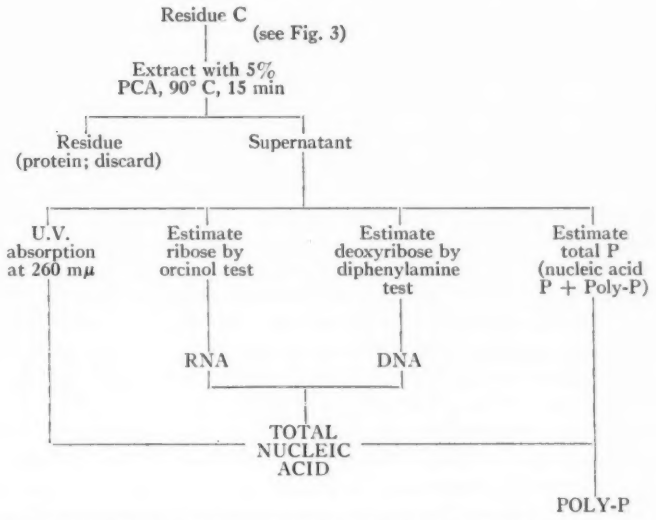

FIG. 4. Modified extraction procedure for the estimation of nucleic acids in *Euglena*.

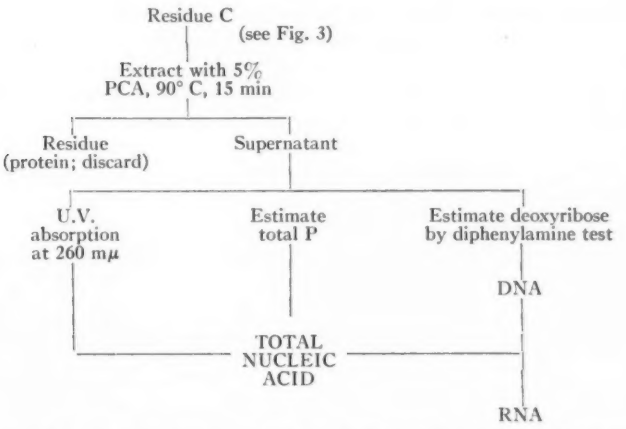

FIG. 5. Modified extraction procedure for the estimation of nucleic acids in pea leaves and beet roots.

The Quantitative Extraction and Separation of RNA Nucleotides

It is often required to extract and separate quantitatively the component nucleotides of plant RNA such as in the determination of RNA base ratios. Part of the procedure outlined in Fig. 3 can serve as a preliminary purification step. The hydrolysis with KOH quantitatively converts RNA to its component nucleotides. These may be then separated either chromatographically,

electrophoretically, or on ion exchange resins, starting with the supernatant of the neutralized KOH hydrolyzate (see Fig. 3). A one-dimensional two-solvent paper chromatographic separation of the four RNA nucleotides was developed. A one-dimensional system is more convenient than a two-dimensional system where a large number of samples are to be run. RNA hydrolyzate was applied near one end of an unwashed Whatman No. 1 paper (50×23 cm). The paper was developed by descending chromatography with Pabst (25) solvent No. 1 (10 ml concentrated ammonia, 329 ml water, 661 ml isobutyric acid; pH 3.7) in a 13×28×52 cm glass tank. After 20 hours at 25° C, the chromatogram was removed from the tank, dried, and the ultraviolet light absorbing spots were located by observing the chromatogram in ultraviolet light (Mineralight, model SL 2537). Three ultraviolet light absorbing spots were obtained. In order of increasing R_f these were a mixture of uridylic and guanylic acids, cytidylic acid, and adenylic acid. The chromatogram was then cut as close to the cytidylic acid spot as possible on its origin side. The half of the paper containing the mixture of uridylic and guanylic acids was placed in another glass tank of the same size and developed in the reverse direction with Pabst (25) solvent No. III (ammonium sulphate 600 g, 1 l. of 0.1 *M* Na phosphate buffer, pH 6.8, 20 ml of *n*-propanol) for 6 hours at 25° C. Three ultraviolet light absorbing spots were obtained. In order of increasing R_f these were guanosine-3-phosphate, guanosine-2-phosphate, and uridylic acid. The former two were barely separated and were eluted together. The compounds were eluted from the chromatograms with 0.1 *N* HCl and their concentrations estimated individually by their ultraviolet light absorption (19). A separation of the four RNA nucleotides by the chromatographic method described above is shown in Fig. 6A.

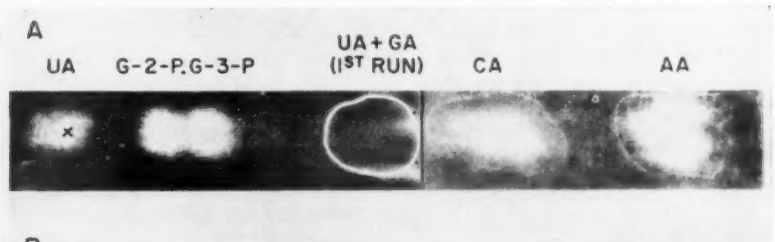

FIG. 6. A one-dimensional separation of RNA nucleotides. A. By paper chromatography (for details see text). B. By anion exchange paper. In the latter case the nucleotides were located and photographed by making an imprint of the developed ion exchange paper (29). AA, adenylic acid; CA, cytidylic acid; GA, guanylic acid; UA, uridylic acid; × indicates the origin.

RNA nucleotides from *Euglena* and *Chlorella* have been also separated on a Dowex-1 anion exchange column (15 cm × 0.8 cm diameter), formate form. The nucleotides were first absorbed onto the resin column and were then successively removed by gradient elution with formic acid following the procedure of Hurlbert *et al.* (10).

A convenient technique for the separation of plant RNA nucleotides is that involving the use of ion exchange paper (33) (for details of this technique see ref. 29). The nucleotides were separated on a paper strip impregnated with a strong anion exchange resin. The method offers certain advantages over the usual procedures which are available for the separation of RNA nucleotides. It is simpler to perform and requires very little apparatus compared with electrophoretic or column chromatographic methods. It is less time consuming than the chromatographic method outlined above, avoids the use of obnoxious solvents, and is subject to less interference by salts. A satisfactory separation of the four RNA nucleotides was obtained on strips of ion exchange paper 1 cm wide. It is hence possible to run a large number of samples simultaneously on a large sheet of paper. Since the nucleotides were absorbed on the ion exchange it was not possible to locate their positions on the paper strip by direct observation or photography in ultraviolet light. The location of the nucleotides was nevertheless rapidly ascertained by making an "imprint" of the developed ion exchange paper (29) and then by observing the "imprint" in ultraviolet light. A separation of uridylic, guanylic, adenylic, and cytidylic acids on ion exchange paper is shown in Fig. 6B. RNA nucleotides isolated from *Euglena*, pea leaves, and barley roots have been separated by this method.

Alternatively base ratio determinations can be carried out by degradation of the nucleic acids to their component bases (20, 31). Such methods have already been applied to plants in a number of instances (3, 9, 15, 18, 21, 32).

Discussion

The investigations conducted on nucleic acid determinations in several plants emphasize the importance of first ascertaining the presence and nature of substances which might interfere in such estimations. Before any existing method for the estimation of nucleic acids is applied to a new plant tissue, the suitability of the method for that particular tissue should first be established. Even in *Euglena* in which the estimation of nucleic acids proved to be less complicated than in the other plants tested, the direct application of existing common methods for nucleic acid determinations introduced certain errors. It is important to remove quantitatively the acid-soluble-P and lipid-P without loss of nucleic acid-P before the nucleic acids are extracted. Loss of nucleic acid-P may eventuate if the concentration of the extracting acid is too strong or if the extraction is prolonged. In the plants tested, pretreatment with an organic solvent such as methanol rendered the acid-soluble compounds more easily extractable. Following the removal of the acid-soluble-P and the lipid-P, the nucleic acids should be quantitatively

extracted. In many instances the Ogur–Rosen method does not quantitatively differentiate between RNA and DNA. Hence, it should not be directly applied to the estimation of nucleic acids in plants except in those tissues for which its suitability has been demonstrated. Methods involving nucleic acid extraction with NaCl solution should only be applied to plants with similar circumspection. On the other hand, the Schneider and Schmidt–Thannhauser nucleic acid extraction methods appear to be quantitative. For this reason they are preferred to the Ogur–Rosen procedure as general methods for the extraction of plant nucleic acids. Although the Schneider and Schmidt–Thannhauser methods quantitatively extract nucleic acids, they are subject to interfering substances during the subsequent estimation of extracted RNA and DNA. Depending on the plant tissue involved and the presence of various impurities, sugar, phosphate, or ultraviolet light absorption determinations may all give false estimations of the nucleic acid concentrations. For the estimation of RNA by the Schmidt–Thannhauser procedure, such interference in the analytical procedures by impurities may be surmounted by the use of an ion exchange column or the still simpler direct addition of ion exchange resin to the RNA extract (7). The results reported in this paper and those of Deken-Grenson and Deken (7) indicate that the Schmidt–Thannhauser procedure, coupled with the use of an anion exchange resin, is the most reliable method for the estimation of nucleic acids in the widest range of organisms.

Acknowledgment

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STUDIES ON FUSARIUM WILT OF BANANAS

V. PATHOGENICITY AND DISTRIBUTION OF F. OXYSPORUM f. CUBENSE RACES 1 AND 2[1]

R. H. Stover and B. H. Waite

Abstract

Two naturally occurring races of the *Fusarium* wilt or Panama disease fungus, *F. oxysporum* f. *cubense* (E.F.S.) Snyd. and Hans., have been identified. Race 1 is world-wide in distribution and attacks certain AAA triploids of *Musa acuminata*, of which variety 'Gros Michel' is the most important, and *Musa textilis*. Race 2 has been identified in Honduras, El Salvador, Puerto Rico, Virgin Islands, and the Dominican Republic and attacks certain ABB triploids of *M. acuminata* × *M. balbisiana* known as 'Chato', 'Bluggoe', 'Rulo', or 'Chamaluco'. Both races are pathogenic on 3- to 4-month-old seedlings of *M. balbisiana*, *M. schizocarpa*, and *M. acuminata* ssp. *banksii*, but the race 2 clone was less pathogenic than the race 1 clones tested. Mature *M. balbisiana* is immune to rhizome infection in the field. 'Odoratum' and 'Inodoratum' cultivars of *F. oxysporum* f. *cubense* are found within race 1; only the 'Inodoratum' cultivar is found within race 2. Circumstantial evidence indicates an independent development of race 2 in different areas. Race 2 wilt-susceptible ABB triploids hybridize and set seed with pollen from diploid *M. balbisiana* (BB) and *M. acuminata* (AA), the two ancestors of the cultivated banana. This will facilitate a study of the inheritance of wilt resistance to *F. oxysporum* f. *cubense* races 1 and 2.

Introduction

In 1953 several acres of a common cooking banana, called 'Chato' or 'Moroca' plantain, were found devastated by *Fusarium* wilt at Choloma near San Pedro Sula, Honduras. Symptoms consisted of bright yellowing of the leaves and characteristic internal vascular discoloration of rhizome and pseudostem. *Fusarium oxysporum* was invariably obtained from the discolored vascular tissue. Since this was the first time the writers had observed *Fusarium* wilt of a hybrid *Musa* triploid (*M. acuminata* Colla × *M. balbisiana* Colla) with an ABB karyotype (10) studies were initiated to determine if a new *Fusarium* race was involved and, if so, its distribution.

Correspondence and a review of literature revealed that *Fusarium* wilt had been reported attacking a cooking banana or plantain in Puerto Rico (2, 9), Virgin Islands, Dominican Republic (3), El Salvador, India (1), and Thailand (8). However, since the common names of banana and plantain varieties change from one locality to another, it was necessary to describe and compare botanically the locally infected variety in order to determine if it was identical with or closely related to varieties reported diseased in other countries. Also, where possible, cultures of diseased tissue were obtained from the different areas. The isolates of *Fusarium* were then compared in culture and on different hosts.

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Contribution from the Plant Pathology Department, Vining C. Dunlap Laboratories, Tela Railroad Co., La Lima, Honduras (a subsidiary of the United Fruit Company).

FIG. 1. A racime of 'Chato' (ABB) showing characteristic bud and position of fruit fingers.

Methods and Results

Identification and Distribution of Varieties of M. acuminata × M. balbisiana (ABB)

In 1926 Pope (7) described the 'Chamaluco' variety which had been introduced to Hawaii from Puerto Rico. This description fitted the infected 'Chato' of Honduras, with the exception of the red ovary of the pistillate and staminate flowers. This minor difference could have been the result of a bud sport. This description (7) and photographs of 'Chato' from Honduras (Figs. 1 and 2) were sent to N.W. Simmonds at the Imperial College of Tropical Agriculture, Trinidad, and to the Federal Experiment Station at Mayaguez, Puerto Rico. The 'Chato' plantain of Honduras was found to be identical with the 'Bluggoe' of Grenada, 'Moko' of Trinidad, 'Whitehouse plantain' of Jamaica, and the 'Chamaluco' of Puerto Rico, all ABB triploids (10). In addition, visiting scientists from El Salvador and the Dominican Republic identified the infected Honduran material as identical with that observed in those countries. It was concluded, therefore, that the 'Chato' plantain of Honduras is identical with or very closely related to other clones of *M. acuminata* × *M. balbisiana* with an ABB karyotype growing throughout the Caribbean area (Table I).

TABLE I

Synonymy of local varietal names of *M. acuminata* × *M. balbisiana* (ABB), a common and important cooking banana of the Caribbean region

Country	Local names	Country	Local names
Honduras,* El Salvador*	'Chato', 'Moroca'	Mexico	'Largo filos', 'Burro filos', Barbaro filos'
Dominican Republic*	'Rulo'	Jamaica	'Whitehall plantain'
Grenada	'Bluggoe'	Cuba	'Burro banana'
Trinidad	'Moko'	Martinique	'Collie'
Puerto Rico,* Virgin Islands*	'Chamaluco', 'Mofofa', 'Piche', 'Malongo', 'Enano', 'Cuatro filos', 'Frying banana'	Guadalupe	'Post'
		St. Lucie	'Mokabon'

*Reported to be attacked by *Fusarium oxysporum* f. *cubense*.

Pathogenicity Studies in Tanks

Single spore isolates of *Fusarium* from 'Chato' and 'Gros Michel' bananas were grown on 10% corn-meal-in-sand cultures. These cultures, when approximately two weeks old, were used to infest chloropicrin-fumigated soil in 35-gal concrete tanks in which were growing triploid edible varieties of *Musa* spp.

In the first pathogenicity test, 'Chato' (ABB), 'Dwarf Cavendish' (AAA), and 'Lacatan' (AAA) bananas were inoculated 5 to 6 weeks after planting. A trench was dug about 4 in. deep around the base of each mat approximately 6 in. from the rhizome. Two hundred grams of the 2-week-old 10% corn-meal-in-sand inoculum were put into the trench and the excavated soil replaced. Check plants received only fungus-free corn-meal-in-sand, and at

no time showed symptoms of *Fusarium* infection. About 50 days after inoculation, when several of the 'Chato' plants began to show aboveground disease symptoms, all rhizomes were removed and examined. The results (Table II) showed that *Fusarium* clone H from 'Chato' was highly pathogenic to 'Chato' but only slightly pathogenic to 'Dwarf Cavendish' and 'Lacatan'. Clones C and D from bananas, which had been shown previously (11) to be pathogenic on 'Gros Michel', were almost non-pathogenic to 'Chato' and 'Dwarf Cavendish' and slightly pathogenic on 'Lacatan'. All clones invaded the 'Lacatan' roots and in some cases entered the rhizome stele, but proliferation in the rhizome stele did not occur. In contrast, clone H from 'Chato' readily invaded the rhizome stele of 'Dwarf Cavendish' and proliferated for several inches outward from the root base.

TABLE II

Pathogenicity of clones of *F. oxysporum* f. *cubense* from *M. acuminata* × *M. balbisiana* (ABB) 'Chato' and *M. acuminata* (AAA) 'Gros Michel' on three varieties of *Musa* growing in concrete tanks containing artificially infested cloropicrin-fumigated soil

Clone	Host source	Host inoculated	% of 10 rhizomes with: Internal pseudostem symptoms	Heavy rhizome infection	Light rhizome infection	No rhizome infection
H	'Chato'	'Chato'*	70	70	20	10
D	'Gros Michel'	'Chato'	0	0	0	100
C	'Gros Michel'	'Chato'	0	0	10	90
H	'Chato'	'Dwarf Cavendish'*	0	0	100	0
D	'Gros Michel'	'Dwarf Cavendish'	0	0	0	100
C	'Gros Michel'	'Dwarf Cavendish'	0	0	0	100
H	'Chato'	'Lacatan'*	0	0	30†	70
D	'Gros Michel'	'Lacatan'	0	0	20†	80
C	'Gros Michel'	'Lacatan'	0	0	30†	70

*Plants of 'Chato', 'Dwarf Cavendish', and 'Lacatan' examined 49, 58, and 56 days after inoculation, respectively.
†Only very light rhizome infection usually involving less than one-half square inch of tissue where infected root entered rhizome stele.

A second tank pathogenicity test was conducted in which eight plants each of 'Chato', 'Dwarf Cavendish', 'Lacatan', and 'Gros Michel' were inoculated with 'Inodoratum' (11) clone C, 'Odoratum' clone D, and 'Inodoratum' clone J from 'Gros Michel' and 'Inodoratum' clone H from 'Chato'. Inoculations were made 8 weeks after planting and the rhizomes were examined 3 months after inoculation. Check plants were free from infection. The results (Table III) show that on 'Chato', clone H from 'Chato' is highly pathogenic but clones D, C, and J from 'Gros Michel', with one exception, were unable to infect the rhizome. On the normally wilt-resistant varieties 'Dwarf Cavendish' and 'Lacatan', clone H from 'Chato' invaded the rhizome but did not pass into the pseudostem, and was much more virulent than the 'Gros Michel' clones. On 'Gros Michel', the 'Chato' clone was capable of invading the rhizome but did not invade the pseudostem, whereas the 'Gros Michel' clones induced heavy rhizome infection and invaded the pseudostem.

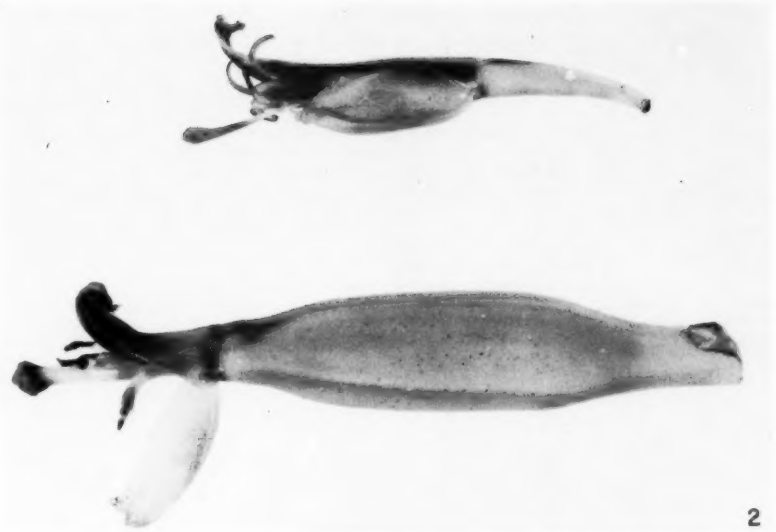

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FIG. 2. Staminate (above) and pistillate (below) flowers of 'Chato' (ABB).
FIG. 3. External symptoms of *Fusarium* wilt on two *M. balbisiana* seedlings induced by 'Inodoratum' clone C from 'Gros Michel' 26 days after inoculation (left). Two plants on the right were inoculated with 'Inodoratum' clone H from 'Chato'.

Among the 'Gros Michel' clones, C induced the most pronounced below- and above-ground symptoms, and aboveground symptoms appeared several weeks earlier than with clones D and J. In general, clone H from 'Chato' was most versatile, invading the rhizome of all four varieties to some extent.

TABLE III

Pathogenicity of clones of *F. oxysporum* f. *cubense* from *M. acuminata* × *M. balbisiana* 'Chato' and *M. acuminata* 'Gros Michel' on eight plants of four *Musa* varieties 3 months after inoculation in concrete tanks

Variety inoculated	*Fusarium* cultivar and clone		No. of inoculated plants showing: Above ground internal symptoms	Heavy rhizome infection	Light rhizome infection	No rhizome infection	Total root traces observed infected at junction with rhizomes
'Chato'	'Inodoratum'	H*	5	7	1	0	36
	'Odoratum'	D*	0	0	1	7	2
	'Odoratum'	C*	0	0	0	8	7
	'Inodoratum'	J*	0	0	0	8	7
'Dwarf Cavendish'	'Inodoratum'	H	0	0	5	3	41
	'Odoratum'	D	0	0	0	8	6
	'Odoratum'	C	0	0	0	8	3
	'Inodoratum'	J	0	0	0	8	1
'Lacatan'	'Inodoratum'	H	0	2	5	1	8
	'Odoratum'	D	0	0	0	8	15
	'Odoratum'	C	0	1	1	6	6
	'Inodoratum'	J	0	0	0	8	0
'Gros Michel'	'Inodoratum'	H	0	2	4	2	38
	'Odoratum'	D	6	4	4	0	84
	'Odoratum'	C	7	7	0	1	130
	'Inodoratum'	J†	3	5	0	2	41

*'Inodoratum' clone H isolated from diseased 'Chato'; 'Odoratum' D and C and 'Inodoratum' J from diseased 'Gros Michel' in Honduras.
†Seven plants only.

Pathogenicity Studies in the Field

One hundred rhizomes of 'Chato' were planted in the field in an area infested with *F. oxysporum* f. *cubense*, and adjacent to 'Gros Michel' that were being rapidly decimated by wilt. Six months after planting, no *Fusarium* wilt had appeared; 20 plants each 12 ft apart in a single row were inoculated by the trenching technique (11) with clone H from 'Chato' and clones C, D, and E from 'Gros Michel'. After 2½ months, 14 of the 20 plants inoculated with clone H from 'Chato' were showing symptoms of disease, especially abundant leaf yellowing. Only the yellow type syndrome (11) has been observed in *Fusarium*-infected 'Chato'. After 3½ months, none of the 'Chato' mats inoculated with *Fusarium* clones from 'Gros Michel' showed disease symptoms.

A second experiment was conducted with isolates of *Fusarium* from ABB triploid hybrids from the Virgin Islands, Honduras, El Salvador, Dominican Republic, and Puerto Rico. In addition, clones C, D, and E from *M. acuminata* (AAA) 'Gros Michel' were inoculated alone and together with clone H from 'Chato'.

These experiments showed (Table IV) that isolates of *Fusarium* from varieties of *M. acuminata* × *M. balbisiana* with the ABB karyotype form a distinct race of *F. oxysporum* f. *cubense*, and that *Musa* clones with this karyotype are not attacked by isolates that are highly virulent on 'Gros Michel'. However, evidence was obtained from field plantings of 'Gros Michel', 'Lacatan', and 'Dwarf Cavendish' that the *Fusarium* from 'Chato' does occasionally infect these varieties. In 1956, 14 rhizomes of 'Lacatan', 19 rhizomes of 'Gros Michel', and 40 rhizomes of 'Dwarf Cavendish' were planted at Choloma in the area where the 'Chato' variety has been almost completely destroyed. Records of wilt incidence were kept in these mats from October 1958 through July 1959. During this time, a total of 13 shoots of 'Lacatan', 13 of 'Gros Michel', and 4 of 'Dwarf Cavendish' showed wilt symptoms. *Fusarium* isolated from these plants appeared identical with that present in the diseased 'Chato' mats scattered throughout the planting. In no case, however, were any of the 'Lacatan', 'Dwarf Cavendish', or 'Gros Michel' mats destroyed; only occasional shoots succumbed and these were usually followed by healthy suckers. These studies indicated that the 'Chato' race of *F. oxysporum* f. *cubense* can occasionally attack 'Gros Michel',

TABLE IV

Pathogenicity of 'Chato' and 'Gros Michel' isolates of *F. oxysporum* f. *cubense* on *M. acuminata* × *M. balbisiana* (ABB) 'Chato' growing in the field

Plot No.	Source of isolate	Host	*Fusarium* cultivar or clone	% of mats diseased*	*Fusarium* cultivar re-isolated
1	Virgin Islands	Plantain†	'Inodoratum'	100	'Inodoratum'
2	Honduras	'Chato'	'Inodoratum' clone H	100	'Inodoratum' clone H
3	El Salvador	'Chato'	'Inodoratum' ropy	100	'Inodoratum' ropy
4	Dominican Republic	'Rulo'	'Inodoratum'	80	'Inodoratum'
5	Puerto Rico	'Chamaluco'	'Inodoratum'	100	'Inodoratum'
6	Virgin Islands	Banana†	'Odoratum'	0	—
7	Honduras	'Gros Michel'	'Inodoratum' clone C	0	—
8	Honduras	'Gros Michel'	'Odoratum' clone D	0	—
9	Panama	'Gros Michel'	'Odoratum' clone E	0	—
10	Honduras	'Chato' + 'Gros Michel'	'Inodoratum' clone H + 'Odoratum' clone D	80	'Inodoratum' ‡
11	Honduras	'Chato' + 'Gros Michel'	'Inodoratum' clone H + 'Inodoratum' clone C	70	'Inodoratum' clone H
12	Honduras + Panama	'Chato' + 'Gros Michel'	'Inodoratum' clone H + 'Odoratum' clone E	80	'Inodoratum' clone H

*Thirteen weeks after inoculation; five mats inoculated in plots numbers 1 to 9 and 10 mats in plots 10 to 12. In mixed inoculation (plots 10 to 12) clones were thoroughly mixed in pails before applying 200 grams of inocula to trench at base of mat.

†Varieties not identified but probably *M. acuminata* × *M. balbisiana* (ABB) 'Chamaluco' and *M. acuminata* AAA) 'Gros Michel'.

‡The rhizome of one plant out of eight sampled yielded a few 'Odoratum' clones along with the 'Inodoratum'.

'Lacatan', or 'Dwarf Cavendish' but that it is not highly pathogenic on these varieties. Also, an occasional shoot of 'Lacatan' shows wilt symptoms in the field as a result of infection with race 1. However, numerous attempts to induce wilt symptoms artificially in mature 'Lacatan' plants by inoculation with different clones, including isolates from diseased 'Lacatan', have been negative. In contrast, *Musa textilis* Née variety 'Bungalanon' was readily infected in the field with race 1 isolates.

Mats of the commercially important and widely grown 'Horn plantain' with an AAB karyotype (10) were frequently present in areas where the 'Chato' (ABB) variety was being destroyed by disease. This variety has never been observed to be infected by *Fusarium* wilt and results with artificial inoculations in the field also were negative.

Pathogenicity Studies with Musa balbisiana and other Musa Seedlings

M. balbisiana (BB) and *M. acuminata* (AA) are considered to be the two wild type seeded diploid progenitors of the triploid cultivated bananas and plantains of commerce (6, 10). *Musa balbisiana* produces numerous seeds and has become wild after its introduction into the American tropics from Australasia. It is found growing vigorously in northern Honduras along rivers and streams, frequently in areas where 'Gros Michel' has been destroyed by *Fusarium* wilt. It is considered immune or highly resistant to *Fusarium* wilt infection and no cases of wilt have ever been recorded in the field, even among mature plants artificially inoculated by the trenching technique.

In 1956, Umali, Ick, and Orillo (13) in the Philippines reported that seedlings of the 'Pacol' clone of *M. balbisiana* were susceptible to *F. oxysporum* f. *cubense*. Three-month-old plants from seed taken from *M. balbisiana* growing in Honduras were also readily infected by isolates of *F. oxysporum* f. *cubense* from 'Gros Michel' (Fig. 3).

Several pathogenicity tests with clone H from 'Chato' and clones C and D from 'Gros Michel' were conducted using *M. balbisiana* seedlings. Seedlings $2\frac{1}{2}$ to 3 months old and 5 to 6 in. tall growing in 6-in. pots of methyl-bromide-fumigated soil were inoculated by digging a small trench about $\frac{1}{2}$ in. from the base of the rhizome. Two grams of a 2-week-old 10% corn-meal-in-sand culture were placed in the trench and the soil replaced. Inoculated pots were placed in an open greenhouse at fluctuating diurnal air temperatures of 70° to 90° F and watered daily except Sunday. Rhizomes were dug and examined 1 month after inoculation. Results of representative experiments are shown in Table V and Fig. 3. Clone C was the most pathogenic, followed closely by clone D from 'Gros Michel'. Clone H from 'Chato', however, was consistently less pathogenic than the 'Gros Michel' *Fusarium* clones. Similar tests were conducted with seedlings of *Musa schizocarpa* Simmonds and *Musa acuminata* ssp. *banksii* from New Guinea and Madras. Again *Fusarium* clone C, followed closely by clone D, was most pathogenic and clone H from 'Chato' was consistently less pathogenic than the two

'Gros Michel' clones. It was concluded that the 'Chato' isolate of *F. oxysporum* f. *cubense* was less pathogenic on seedlings of *M. balbisiana*, *M. schizocarpa*, and *M. acuminata* ssp. *banksii* than the isolates from 'Gros Michel' tested.

TABLE V

Response of *M. balbisiana* seedlings to inoculation with three different clones of *F. oxysoprum* f. *cubense*

Clone	Host source	Disease rating		Average % of plants infected	
		Experiment		Experiment	
		1*	2†	1	2
C	'Gros Michel'	2.45 ± .025	12.8 ± .75	85	93
D	'Gros Michel'	0.40 ± .005	7.3 ± .88	25	73
H	'Chato'	0.25 ± .015	4.6 ± 1.47	10	40

*Disease rating on 20 plants in four blocks of five each 30 days after inoculation. Disease severity scored as follows: 3=severe rhizome vascular discoloration; 2=moderate; 1=slight.
†Disease rating on 30 plants in six blocks of five each 30 days after inoculation. Disease severity scored as follows: 4=external aboveground symptoms; 3=severe rhizome vascular discoloration; 2=moderate; 1=slight.

Characteristics and Distribution of F. oxysporum f. cubense Races 1 and 2

A race of *F. oxysporum* f. *cubense* may be defined as one or more clones which may be distinguished from other clones by pathogenic potential, either quantitative or qualitative, on a series of differential hosts (11). Results of pathogenicity tests showed that there were two such races of *F. oxysporum* f. *cubense* (Table VI). *F. oxysporum* f. *cubense* race 1 attacks 'Gros Michel' bananas, wherever they are grown, as well as several other triploid edible varieties of *M. acuminata*, and abaca (*M. textilis*) (2, 8, 11, 14, 15). Race 1 does not attack clones of *M. acuminata* × *M. balbisiana* (ABB). *F. oxysporum* f. *cubense* race 2 attacks clones of *M. acuminata* ×

TABLE VI

Relative resistance or susceptibility of five *Musa* hosts to *F. oxysporum* f. *cubense* races 1 and 2

Race	Variety and karyotype				
	'Gros Michel', AAA	'Chato', ABB	'Dwarf Cavendish', AAA	'Lacatan', AAA	*M. balbisiana*, BB
	Field response				
1	Susceptible	Highly resistant	Highly resistant	Highly resistant	Immune
2	Resistant	Highly susceptible	Resistant	Resistant	Immune
	Tank or pot response				
1	Susceptible	Highly resistant	Highly resistant	Highly resistant	Susceptible
2	Resistant	Highly susceptible	Resistant	Resistant	Slightly resistant

M. balbisiana with the ABB karyotype (10) and is only weakly pathogenic on *M. acuminata* (AAA) 'Gros Michel'. Race 1 has a world-wide distribution (2, 8, 15), while the known distribution of race 2 is outlined in Table VII. It is not definitely known if the reports of *Fusarium* wilt on varieties of cooking banana in India and Thailand involve race 2. Efforts are now being made to compare the host and fungus from the Far East and the Hawaiian Islands with those from the Caribbean.

TABLE VII

Known and probable distribution of *Fusarium oxysporum* f. *cubense* race 2 on *M. acuminata* × *M. balbisiana* (ABB) variety 'Bluggoe', 'Chato', 'Chamaluco', or 'Rulo'

Country	Locality	Year reported	Reported by	Common local name of variety	Race 2 verified in this report
Puerto Rico	Throughout island	1919 1923 1930	Brandes (2) Tucker (12) Rios (9)	'Chamaluco' 'Piche'	Yes
Virgin Islands	Islands wide	1930 (?)	This report	'Frying banana'	Yes
Honduras	San Pedro Sula, Ulua Valley	1953	Waite (unpublished)	'Chato', 'Moroca'	Yes
El Salvador	San Luis Talpa	1956	Waite (unpublished)	'Moroca'	Yes
Dominican Republic	Caño Grande	1958	Castellani (3) This report	'Rulo'	Yes
India	Maduro district, Madras	1929	Ayyangar (1)	'Chinna Mondan'	No
Thailand	Bang Sorn Bang Noi	1934	Reinking (8)	'Nam Wa'	No

M. acuminata × *M. balbisiana* (ABB) is grown widely in Guatemala, Costa Rica, Panama, and Colombia. No disease has been found in these areas. Also the disease is reported absent in Jamaica and Trinidad (5). Most 'Chato' plantings in the Ulua Valley of Honduras are still disease-free. However, the disease is spreading outward from the original centers of infections around Choloma and San Pedro Sula. Isolated 'Chato' plantings as far as 30 miles from the original outbreak are now infected. 'Chato' is usually grown in small plantings from a few mats to several acres around farmsteads and towns and most plantings are isolated by from a few to many miles. Movement of infected planting stock no doubt accounts for some of the disease spread; if this could be prevented, the natural isolation of most 'Chato' plantings would greatly hinder the eventual destruction of this important food crop in the American tropics.

Previous studies (11) showed that *F. oxysporum* f. *cubense* exists in diseased rhizomes of 'Gros Michel' bananas in two major clonal groups or cultivars designated 'Odoratum' and 'Inodoratum'. All isolates of *Fusarium* from naturally infected ABB hybrids have been of the 'Inodoratum' cultivar even in areas where the 'Odoratum' cultivar was known to be present. Forty-five mats of 'Chato' were inoculated with mixed cultures of the 'Chato' 'Inodoratum' clone H and 'Gros Michel' 'Odoratum' clones D and E and 'Inodoratum' clone C. In all cases but one the 'Inodoratum' clone H from

'Chato' was recovered from the diseased rhizomes and pseudostems (Table IV). In the single exception, a few 'Odoratum' colonies developed along with the 'Inodoratum' from diseased rhizome tissue.

Discussion

Brandes in 1919 (2) was the first to report the presence of *Fusarium* in the 'Chamaluco' variety in Puerto Rico and he stated that isolates of the fungus from Puerto Rico were all of the 'Inodoratum' cultivar. These findings are in agreement with results of our isolations from 'Chamaluco' in Puerto Rico and other areas in that no 'Odoratum' isolates have yet been obtained from a naturally infected ABB hybrid. Tucker (12) states the disease was present as early as 1916 in Puerto Rico. Rios (9) reported that *Fusarium* has almost eliminated this variety as an important food plant. Only in occasional isolated locations on the island are a few healthy plants still found.

All evidence indicates that *F. oxysporum* f. *cubense* race 2 has evolved independently in several locations. The 'Chato' variety has been present in Central America for at least 50 years but diseased plants were first noted in 1953 and probably had been present for no more than 5 years. Thus the history of the disease goes back no further than 1940 in Central America and about 1910 in Puerto Rico, assuming disease may be present 5 to 7 years before becoming sufficiently widespread to be noticed. The frequency with which the disease is now appearing in Honduras suggests that if it was present before 1940 it would likely have been noticed. If evidence for the presence in India (8) and Thailand (1) of race 2 is confirmed, this will be a further indication of the independent origin of race 2. Banana varieties and *M. textilis* have been moved from one country to another into the commercial banana plantations, but the earliest known center of 'Chato' infection in Honduras was in an area that had not grown bananas commercially since 1920 and is noted for cattle ranching. Only recently has a center of infection appeared among the numerous 'Chato' plantings in the commercial banana-growing area. It is unlikely, therefore, that race 2 has been introduced in infected *Musa* planting stock, consisting mostly of the 'Gros Michel' and other edible AAA triploids.

The presence of two races of *F. oxysporum* f. *cubense* attacking *Musa* triploids with AAA and ABB karyotypes offers a valuable tool in elucidating the inheritance of *Fusarium* wilt resistance in *Musa* spp. With increased emphasis on breeding for disease resistance as the ultimate solution to the banana disease problem, a knowledge of the inheritance of disease resistance and susceptibility is of prime importance. The 'Chato' or 'Bluggoe' variety of *M. acuminata* × *M. balbisiana* (ABB) readily crosses with *M. acuminata* (AA) or *M. balbisiana* (BB) as the pollen parent and seed set is from fair to good (4). Studies are now underway to determine the response of progeny from such crosses to races 1 and 2 of *F. oxysporum* f. *cubense*. The detection and identification of races of *F. oxysporum* f. *cubense* will undoubtedly play an important role in the development of wilt-resistant bananas through breeding.

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DISTRIBUTION AND CYTOLOGY OF ELYMUS MACOUNII VASEY[1]

A. T. H. Gross

Abstract

The distribution of *Elymus macounii* Vasey in the Prairie Provinces of Canada is extensive and apparently governed by the occurrence of its putative parents, *Agropyron trachycaulum* (Link) Malte and *Hordeum jubatum* L. The hybrid *E. macounii* was produced by controlled cross-fertilization of *A. trachycaulum* and *H. jubatum*. Subsequently, octoploid *E. macounii* was obtained by colchicine treatment. Cytological studies indicated $2n = 28$ for *A. trachycaulum*, *H. jubatum*, and *E. macounii* (both artificially produced and naturally occurring hybrids). Irregular meiosis of *E. macounii* and some irregularity in meiosis of octoploid *E. macounii* were observed.

Introduction

In the Prairie Provinces of Canada and in areas of the United States (Stebbins *et al.* (15), and Boyle and Holmgren (3)) sterile *Elymus macounii* Vasey has been observed to occur naturally. The widespread distribution of this sterile plant would indicate that it is of hybrid origin. Booher and Tryon (2) found that *E. macounii* in Minnesota was sterile and that it fit a hybrid interpretation. It was postulated by Stebbins *et al.* (15) that *E. macounii* arose from the cross *Agropyron trachycaulum* (Link) Malte with *Hordeum jubatum* L. Subsequently Forsberg (6), and Boyle and Holmgren (3), reported successful artificial production of *E. macounii*.

Cytological studies of *A. trachycaulum* and *H. jubatum* have been reported by several authors; however, very few reports have been made on *E. macounii*. The $2n$ chromosome number of 28 for *A. trachycaulum* was reported by Hartung (8), Nielsen and Humphrey (9), Peto (10), and Stebbins *et al.* (15). Aase and Powers (1), Chin (5), and Quincke (11) found a $2n$ number of 28 for *H. jubatum*. Three reports of $2n = 28$ for *E. macounii* were made by Brown (4), Nielsen (8), and Boyle and Holmgren (3). Stebbins *et al.* (15), and Boyle and Holmgren (3), in studies of meiosis of *E. macounii* observed irregularity in metaphase I and anaphase I. Meiosis of octoploid *E. macounii* has not previously been reported.

This study was undertaken to determine the distribution of *E. macounii* in the Prairie Provinces of Canada, to produce hybrid and octoploid *E. macounii* and to observe their meiosis and mitosis.

Materials and Methods

Distribution of natural hybrids of *E. macounii* was determined by examination of herbarium specimens and by survey of native vegetation in the Saskatoon, Saskatchewan, area. Information concerning the distribution of *E. macounii* was provided from herbaria at the following locations: Botany and

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Plant Pathology Division, Canada Department of Agriculture, Ottawa, Ontario; University of Manitoba, Winnipeg, Manitoba; Dominion Experimental Farm, Brandon, Manitoba; Regina College, Regina, Saskatchewan; University of Saskatchewan, Saskatoon, Saskatchewan; Dominion Experimental Farm, Swift Current, Saskatchewan; Canada Agriculture Research Laboratory, Saskatoon, Saskatchewan; and University of Alberta, Edmonton, Alberta. The survey of native vegetation consisted of examination of depressional areas with *H. jubatum* as the dominant species.

Parental plants used for the production of *E. macounii* consisted of collections of native *H. jubatum* and strains of *A. trachycaulum* under test at the Canada Agriculture Research Laboratory, Saskatoon, Saskatchewan. The *E. macounii* plants under study consisted of native collections of the sterile hybrid obtained from the Saskatchewan districts of: Dana, Humboldt, Meath Park, St. Gregor, Saskatoon, Vonda, and Watson. The artificially produced hybrids of *E. macounii* were from crosses of *A. trachycaulum* and *H. jubatum* made at Canada Agriculture Research Laboratory, Saskatoon, Saskatchewan, in 1949 and 1953. Octoploid *E. macounii* was obtained by colchicine treatment in 1947–1948.

Studies of meiosis and mitosis were conducted on 15 plants of *A. trachycaulum* 13 plants of *H. jubatum*, 10 plants of *E. macounii*, and 12 plants of octoploid *E. macounii*. Both meiotic and mitotic stages were studied using acetocarmine techniques.

Experimental Results

Collections of *E. macounii* were made at numerous sites in the grassland and parkland of the Prairie Provinces of Canada as indicated in Fig. 1. *Agropyron trachycaulum* and *H. jubatum* were associated with *E. macounii* at 13 sites surveyed in the Saskatoon, Saskatchewan, area. From herbaria *A. trachycaulum* and *H. jubatum* were indicated as associated plants at 22 locations at which *E. macounii* was collected. The frequent occurrence of *A. trachycaulum* and *H. jubatum* in association with sterile *E. macounii* indicates frequent and widespread hybridization between the parental species.

Artificially produced hybrids between *A. trachycaulum* and *H. jubatum* were obtained in 1949 and 1953. The hybrids are morphologically intermediate between the two parents (Figs. 2 and 3). Using pollen from *H. jubatum* on hand emasculated *A. trachycaulum* 21 seeds were obtained in 1949 and 23

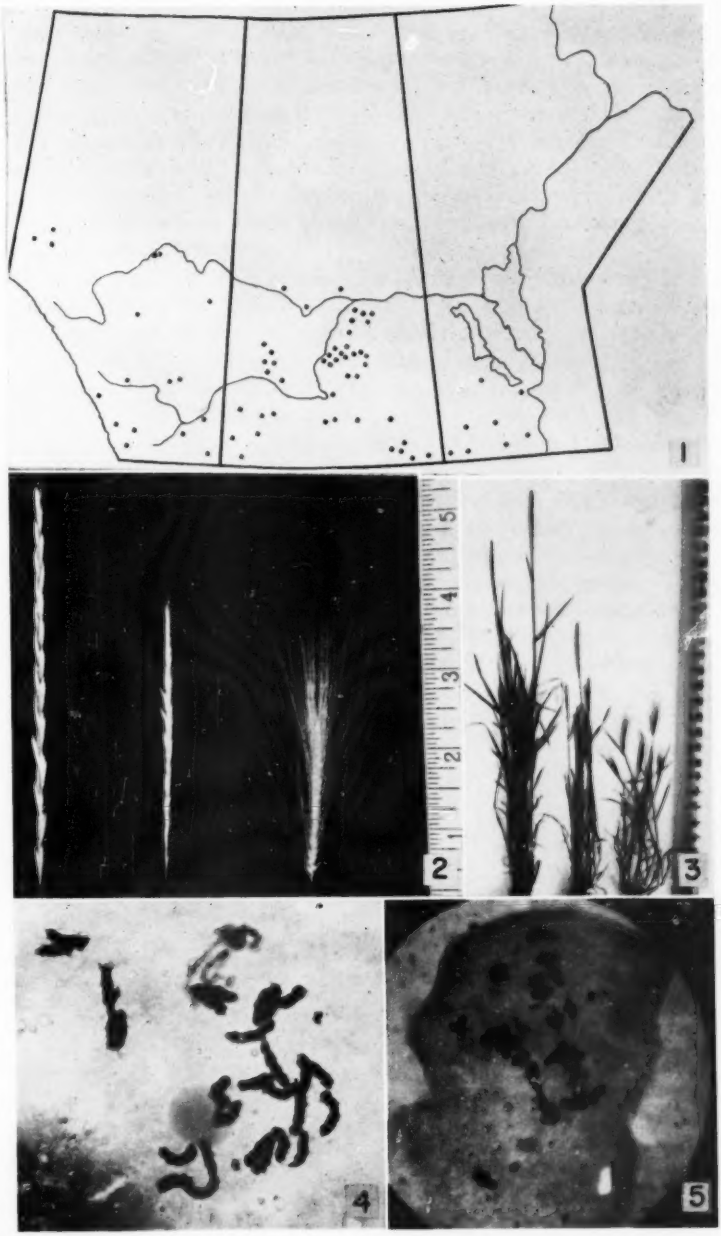
FIG. 1. Locations from which *Elymus macounii* has been reported in Alberta, Saskatchewan, and Manitoba.

FIG. 2. Spikes of *Agropyron trachycaulum* (left), *Elymus macounii* (center), and *Hordeum jubatum* (right). Note the length of spike and amount of awning.

FIG. 3. Plants of *Agropyron trachycaulum* (left), *Elymus macounii* (center), and *Hordeum jubatum* (right). Note differences in culm height and leafiness.

FIG. 4. Diakinesis in *Elymus macounii* meiosis showing slight chromosomal association. Approximate magnification ×2400.

FIG. 5. First metaphase in octoploid *Elymus macounii* showing good chromosomal association. Approximate magnification ×2000.



Gross—Can. J. Botany

seeds were obtained in 1953. From the one reciprocal cross attempted in 1953 one seed was obtained. Five plants were successfully grown from the 1949 crosses and six plants were obtained from the 1953 crosses. The seed derived from pollination of *H. jubatum* by *A. trachycaulum* was viable. All of the artificial hybrids were sterile and resembled natural hybrids of *E. macounii*. Successful production of *E. macounii* by crosses between *A. trachycaulum* and *H. jubatum* using either as pollen parent, reported here and by Boyle and Holmgren (3), would indicate that natural hybrids can occur with either species as maternal parent.

Cytological examination of two root-tip cells for each of 15 plants of *A. trachycaulum*, 10 plants of *E. macounii*, and 13 plants of *H. jubatum* revealed $2n$ chromosome numbers of 28. The somatic chromosome number of octoploid *E. macounii* was difficult to determine because of the large number of chromosomes in each root-tip cell studied. The $2n$ number as estimated from two cells of each of 12 plants was 56.

In meiotic studies of *A. trachycaulum* and *H. jubatum* 14 bivalents were observed regularly at metaphase I, no multivalent associations were noted but micronuclei occurred in a number of tetrads (Table I). The meiotic behavior of artificial and natural hybrids of *E. macounii* was irregular. In both, chromosome association was reduced (Fig. 4) and separation at anaphase I was irregular as shown by lagging chromosomes and chromosome bridges (Table I). In octoploid *E. macounii* difficulty was experienced in observing clearly defined bivalents at metaphase I (Fig. 5). No univalents were recorded, but groups of eight or more were observed frequently. Groups were interpreted as being a result of clumping rather than multivalent associations, because in the 47 cells in which chromosome clumping was not observed there were no multivalent associations recorded. Anaphase I was irregular as shown by lagging chromosomes and chromosome bridges, and by micronuclei observed in the tetrads (Table I).

TABLE I

Meiotic behavior of *Agropyron trachycaulum*, *Hordeum jubatum*, and *Elymus macounii* at metaphase I, anaphase I, and tetrad stages of development

	Metaphase I				Anaphase I		Tetrad	
		Mean no. chromosome associations per cell						
	Cells	I	II	IV	Cells	L*	Cells	M*
A. trachycaulum	199	0.04	13.98	0.0	40	0	746	0.40
H. jubatum	141	0.00	14.00	0.0	89	0	120	4.17
E. macounii								
Natural	340	20.76	3.61	0.005	—	—	43	100.00
Artificial	138	18.96	4.52	0.0	135	100	298	100.00
Octoploid	47	0.00	28.00	0.0	205	4.8	108	27.80

*L = per cent cells containing lagging chromosomes.
M = per cent tetrads with micronuclei.

Discussion

The results reported here indicate that *Elymus macounii* is a hybrid of the species *Agropyron trachycaulum* and *Hordeum jubatum*. This is in agreement with the work reported by Boyle and Holmgren (3), and corroborates the postulate of Stebbins *et al.* (15) regarding the parentage of *E. macounii*.

Two explanations for the widespread distribution and prevalence of *E. macounii* in native vegetation are suggested: (1) as *E. macounii* is sterile and apparently short-lived, there must be frequent natural crossing between *A. trachycaulum* and *H. jubatum*, and (2) natural tolerance of *E. macounii* to salinity and flooding permits it to successfully compete with seed-bearing associates.

In the artificial production of *E. macounii* the seed parent was usually *A. trachycaulum*, because its florets are larger and easier to emasculate than those of *H. jubatum*. The rachis of *H. jubatum* disarticulates readily further restricting its use as seed parent. Reciprocal crosses between *A. trachycaulum* and *H. jubatum* resulted in successful production of *E. macounii* which indicates that either could serve as seed parent.

Close generic relationships exist among *Agropyron*, *Elymus*, and *Hordeum* as indicated by interfertility data. This observation is in agreement with the findings of Stebbins *et al.* (12, 13, 14, 15). In the Prairie Provinces of Canada *Agropyron*, *Elymus*, and *Hordeum* are widely distributed. Further crosses involving the 10 *Agropyron*, 8 *Elymus*, and 3 *Hordeum* which are native to the Canadian Prairie Provinces would aid in clarifying their species and generic relationships.

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THE CYTOLOGY OF THREE HYBRIDS INVOLVING HORDEUM JUBATUM L.: THE CHIASMA DISTRIBUTIONS AND THE OCCURRENCE OF PSEUDO RING-BIVALENTS IN GENETICALLY INDUCED ASYNAPSIS[1]

E. B. WAGENAAR[2]

Abstract

Three hybrids, two of *Hordeum jubatum* L. × *Secale cereale* L. var. Prolific, and one of *H. jubatum* × *H. bulbosum* L., were examined cytologically. The two genomes of *H. jubatum* showed autosyndesis in the *H. jubatum* × *S. cereale* hybrids. It was concluded, therefore, that *H. jubatum* has two closely related genomes.

One of the *H. jubatum* × *S. cereale* hybrids displayed weak asynapsis. The *H. jubatum* × *H. bulbosum* hybrid, on the other hand, showed strong asynapsis, although the prophase pairing was almost complete. It was concluded that the asynapsis of this latter hybrid was due to interaction of genes of the *H. jubatum* and the *H. bulbosum* genomes.

The chiasma distributions of the hybrids were tested for randomness by Gaul's formula. It was found that the chiasma distributions of the *H. jubatum* × *S. cereale* hybrids followed a random pattern, whereas in the *H. jubatum* × *H. bulbosum* hybrid the distribution was disturbed by a secondary effect. Cytological observations and theoretical considerations led to the conclusion that the occurrence of pseudo ring-bivalents had disturbed the apparent chiasma distribution in the *H. jubatum* × *H. bulbosum* hybrid. A theory of the origin of pseudo bivalents and an evaluation of Gaul's formula is presented.

Introduction

In a previous paper the cytology of two hybrids involving *Hordeum jubatum* L. ($2n=28$) and *Secale cereale* L. ($2n=14$) was described (27). In these 21-chromosome hybrids the seven *S. cereale* chromosomes could be distinguished from the 14 *H. jubatum* chromosomes by their larger size. It was observed that the *H. jubatum* chromosomes formed three to seven bivalents autosyndetically with a mode at six bivalents per cell. One of the hybrids (No. 33) had an average of 5.9 bivalents and 9.1 univalents per cell.

The strong autosyndetic behavior of the *H. jubatum* chromosomes contrasted sharply with the very low formation of bivalents in the hybrid *H. jubatum* × *H. vulgare* L. observed by Kerber (7). The latter hybrid had a somatic number of 21 chromosomes of which 14 were also from *H. jubatum*. Kerber observed from none to four bivalents, mostly open, with none to two occurring most frequently. Similar low bivalent frequencies in the hybrids involving the same species were reported by Rajhathy and Morrison (19). The great difference in the formation of bivalents between the hybrids of *H. jubatum* × *S. cereale* and *H. jubatum* × *H. vulgare* can be attributed to three possibilities. Firstly, differences in environmental conditions during

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growth of the hybrids; secondly, differences with respect to autosyndetic pairing between *H. jubatum* strain "E" collected at Edmonton and "S" collected at Saskatoon, used by the author and Kerber respectively; and thirdly, the presence of genes or gene combinations inhibiting autosyndetic pairing in the hybrid *H. jubatum* × *H. vulgare*.

To determine which of these is the most likely cause of the differences, the author made some additional crosses between *H. jubatum* and *S. cereale*, in which Kerber's source of *H. jubatum* was used. The resulting two hybrids were comparable with the *H. jubatum* × *H. vulgare* hybrid, since they all contained *Hordeum* chromosomes from the same parental source.

A third hybrid between *H. jubatum* and *H. bulbosum* was obtained and will also be discussed in this paper. Since *H. bulbosum* ($2n=28$) is an autotetraploid (1, 11), it was hoped that a high number of autosyndetic bivalents would result in partial fertility. However, the hybrid was asynaptic[3] and completely sterile.

In the present paper, which deals with the cytology of these three hybrids, the following problems have been studied.

1. Bivalent formation at metaphase I considered in relation to those of Kerber's *H. jubatum* × *H. vulgare* hybrid and the previously reported hybrid of *H. jubatum* × *S. cereale* var. Antelope (27).

2. Chiasma distributions at metaphase I. A deviation from the random distribution was found in the asynaptic hybrid *H. jubatum* × *H. bulbosum*, and a search was made for the origin of this discrepancy.

Materials and Methods

All crosses involved one *H. jubatum* (S) plant grown from seed collected by Dr. E. R. Kerber at Saskatoon, Saskatchewan. The pollen sources used were *Secale cereale* var. Prolific (spring habit) and *H. bulbosum*, collected by Dr. L. P. V. Johnson in Syria, who kindly provided the seeds.

The hybrids were obtained with the aid of embryo culture. This technique was previously described (27). The pollen mother cells were fixed in Carnoy's A solution, and the cytological observations were made on squash preparations stained with acetocarmine.

For studies of chiasma distributions in the hybrids each attachment between chromosome arms at metaphase I was considered to comprise one chiasma: a rod bivalent was considered to have one chiasma, a ring bivalent two chiasmata, and so forth. This method was used since most bivalents had terminalized chiasmata. The chiasma distributions were tested by the formula, $p = (X^2 + X - B)/((2X - B).Z)$, developed by Gaul (4, 5) for asynaptic plants.

[3]Since the meiotic prophase of the hybrid *H. jubatum* × *S. cereale* No. 727 was not studied, the actual cause of the greater incidence of univalents at metaphase I of this hybrid was not cytologically determined. Therefore, the word "asynapsis" is used throughout this paper, despite the fact that most of the univalents at metaphase I of the *H. jubatum* × *H. bulbosum* hybrid resulted from desynapsis.

Results

Embryo Culture

From the cross *Hordeum jubatum* (S) × *Secale cereale* var. Prolific, 190 embryos were cultured of which five germinated. Only two embryos formed coleoptiles as well as roots and developed into healthy hybrid plants (No. 660 and 727). The low percentage of germination was mainly due to the high degree of fungal and bacterial contamination encountered in this set of crosses, in spite of surface sterilization of the embryos. This probably resulted from the fact that the crosses were made in the field; when the crosses were made in the greenhouse the incidence of contamination was usually much lower.

From the cross *H. jubatum* (S) × *H. bulbosum* one hybrid plant was obtained from 20 cultured embryos. The plant was very weak, and although it developed spikes and permitted cloning, much difficulty was encountered in maintaining it. Clones transplanted to the field in the spring did not survive.

Cytology of the Hybrids

The observed meiotic features of the three hybrids will be presented separately. Since the interest of this study is mainly centered on problems concerning pairing, chiasma and bivalent formation, meiotic stages later than metaphase I will not be discussed.

1. *H. jubatum* (S) × *S. cereale* var. Prolific, No. 660

Table I shows the frequencies of the various cell categories at metaphase I as observed in the primary florets. Observations on metaphase cells obtained from secondary florets were omitted, since they showed a much greater variability in bivalent and univalent formation and had a markedly lower average number of bivalents per cell.

The cytological features of the metaphase I cells of this hybrid were very similar to those of hybrid No. 33 reported previously (27). The *Secale*

TABLE I

Chromosome associations and their frequencies at metaphase I in primary florets of the hybrid *Hordeum jubatum* (S) × *Secale cereale* var. Prolific, No. 660

Chromosome associations			Frequency		Average number of bivalents per cell*		Chiasmata per bivalent*
I	II	III	No. of cells	%	Open	Closed	
19	1		1	0.5	—	—	—
17	2		1	0.5	—	—	—
15	3		2	0.9	—	—	—
13	4		11	5.0	1.81	2.18	1.54
11	5		44	19.9	2.39	2.61	1.52
9	6		93	42.1	2.91	3.09	1.52
7	7		60	27.1	3.38	3.62	1.52
5	8		3	1.4	—	—	—
10	4	1	1	0.3	—	—	—
8	5	1	5	2.3	2.40	2.60	1.52
Total cells:			221	Averages:	2.87†	3.03†	1.52†

*Shown only for categories with five or more cells.
†Values were calculated for all categories.

chromosomes, being larger, were readily distinguishable, and the *Hordeum* chromosomes exhibited strong autosyndesis (Fig. 1). The average number of bivalents and univalents per cell was 5.9 and 9.1, respectively, which is exactly the same as that of hybrid No. 33 (27). Since both hybrids (No. 33 and No. 660) were obtained from two different *H. jubatum* strains there was apparently no difference in the autosyndetic pairing capacities of their haploid set of chromosomes. Only the average number of chiasmata per bivalent was slightly lower in the present hybrid (1.52 compared with 1.59 for hybrid No. 33 (27)).

The frequency of bivalents autosyndetically formed by the *Secale* chromosomes was one in 30 cells, thus approximating earlier observations (13, 27). A few *Secale–Hordeum* bivalents were also recorded (one in 43 cells; Fig. 1). Furthermore, a trivalent was observed in each of seven cells; five consisted of *Hordeum* chromosomes only, one of *Secale* chromosomes only, and one consisted of one *Hordeum* and two *Secale* chromosomes.

2. *H. jubatum* (S) × *S. cereale* var. Prolific, No. 727

This hybrid showed some differences in bivalent formation at metaphase I in comparison with the preceding hybrid. For this study only pollen mother cells of anthers of first florets were examined cytologically. The results of an analysis of 300 cells are presented in Table II.

TABLE II

Chromosome associations and their frequencies at metaphase I of the hybrid *Hordeum jubatum* (S) × *Secale cereale* var. Prolific, No. 727

Chromosome associations			Frequency		Average number of bivalents per cell*		Chiasmata per bivalent*
I	II	III	No. of cells	%	Open	Closed	
21			2	0.7	—	—	—
19	1		7	2.3	0.71	0.29	1.29
17	2		20	6.7	1.45	0.55	1.28
15	3		42	14.0	2.38	0.62	1.21
13	4		79	26.3	2.85	1.15	1.29
11	5		77	25.7	3.64	1.36	1.27
9	6		46	15.3	4.20	1.80	1.30
7	7		21	7.0	4.57	2.43	1.35
12	3	1	3	1.0	—	—	—
10	4	1	3	1.0	—	—	—
Total cells:			300	Averages:	3.15†	1.24†	1.28†

*Shown only for categories with five or more cells.
†Values were calculated for all categories.

These data show that the variation in the number of bivalents per cell is much greater than that observed in hybrids No. 33 (27) and No. 660. The range was from 21 univalents to seven bivalents and seven univalents with a mode at four bivalents per cell (Fig. 2). The average number of chromosome associations per cell was 4.4 bivalents and 12.1 univalents. This average

number of bivalents is 1.5 lower than the combined average number of the other two hybrids. Furthermore, the average number of chiasmata (showing a slight fluctuation among the cell categories) was much lower, having a value of 1.28. The average frequency of open bivalents (71.6%) was, therefore, much higher than in the other two hybrids.

3. *Hordeum jubatum* (S) × *Hordeum bulbosum*, No. 295

It is noteworthy that this hybrid had a somatic chromosome number of 27 instead of the expected 28. This unexpected chromosome number may have originated as follows. *H. bulbosum* is an autotetraploid which regularly forms several multivalents per cell (1, 11). Occasionally, therefore, gametes may occur that have 13 chromosomes instead of the normal number of 14. It is possible that such a male gamete fused with a normal *H. jubatum* female gamete and gave rise to this hybrid plant.

Since *H. bulbosum* is an autotetraploid it is to be expected that its two genomes in the haploid condition regularly pair autosyndetically. This expectation was confirmed by the observations of Konzak *et al.* (10) and Morrison and Rajhathy (12) in the hybrids *H. bulbosum* × *H. vulgare*. The *H. jubatum* chromosomes in the haploid condition can also form autosyndetically three to seven bivalents (Table I; ref. 27). In the hybrids of these two species, therefore, a range of 10 to 14 bivalents may usually be expected.

To investigate chromosome pairing, cells in pachytene, diplotene, and diakinesis were studied. Pairing was often complete and several cells at diakinesis contained 13 pairs, the maximum possible in this hybrid (Fig. 3). In other cells, however, a maximum of nine unpaired chromosomes was observed. If these unpaired chromosomes are assumed to be from *H. jubatum*, then the degree of pairing of the chromosomes in meiotic prophase agrees very well with that expected.

Although the chromosomes occurred in pairs at diakinesis, several failed to remain together due to lack of chiasmata (Fig. 3). Other pairs had one or two chiasmata which gave rise to open or closed bivalents at metaphase I. Still other pairs remained together due to "stickiness". At diakinesis the chromosomes of these pairs were attached at one or both ends, although true chiasmata were lacking. Such pairs would show "side-by-side" or "end-to-end" associations (following the terminology of Person (16)) at metaphase I (Figs. 3, 4, 6, 7, and 8).

The results of the study on the metaphase I cells of this hybrid were quite different from that expected (Table III). There was a range of zero to seven bivalents per cell with a mode of four. The average number of associations for all cells was 3.7 bivalents and 19.6 univalents per cell. The average number of bivalents per cell in this hybrid was thus less than that observed in the two *H. jubatum* × *S. cereale* hybrids. Most of the bivalents were open (69.5%) because of the low average number of chiasmata per bivalent (1.30). The values of certain cell categories deviated considerably from this average (Table III).

TABLE III

Chromosome associations and their frequencies at metaphase I of the hybrid *Hordeum jubatum* (S) × *Hordeum bulbosum*, No. 295

Chromosome associations			Frequency		Average number of bivalents per cell*		Chiasmata per bivalent*
I	II	III	No. of cells	%	Open	Closed	
27			4	3.1	—	—	—
25	1		11	8.7	0.82	0.18	1.18
23	2		15	11.8	1.40	0.60	1.30
21	3		18	14.2	2.06	0.94	1.32
19	4		36	28.3	2.86	1.14	1.29
17	5		27	22.3	3.52	1.48	1.30
15	6		8	6.3	3.25	2.75	1.46
13	7		6	4.7	5.33	1.67	1.25
20	2	1	1	0.8	—	—	—
18	3	1	1	0.8	—	—	—
Total cells:			127	Averages:	2.57†	1.12†	1.30†

*Shown only for categories with five or more cells.
†Values were calculated for all categories.

Analytical Studies

The cytological data as presented in the preceding section can now be compared and analyzed. Table IV summarizes the meiotic behavior of the chromosomes observed in the three hybrids. Also included are pertinent data on the hybrids *H. jubatum* (E) × *S. cereale* var. Antelope, No. 33 (27) and *H. jubatum* (S) × *H. vulgare* var. Hannchen (7).

The hybrids *H. jubatum* × *S. cereale* No. 33 and 660, although obtained from two different *H. jubatum* strains, demonstrated the general ability of the *H. jubatum* chromosomes to pair autosyndetically. The average number of autosyndetic bivalents was lower in the remaining three hybrids, despite the fact that in the *H. jubatum* × *H. bulbosum* hybrid, the *H. bulbosum* chromosomes can also pair autosyndetically. Furthermore, the average number of chiasmata per bivalent was lower.

FIGS. 1–8. Magnification 600×

FIG. 1. *H. jubatum* × *S. cereale*, No. 660. Metaphase I: 6 bivalents of *Hordeum* chromosomes, 1 bivalent of a *Hordeum* and a *Secale* chromosome, and 7 univalents of which 6 are *Secale* chromosomes.

FIG. 2. *H. jubatum* × *S. cereale*, No. 727. Metaphase I: 3 bivalents, 1 pseudo bivalent (arrow), 13 bivalents of which 7 are *Secale* chromosomes.

FIG. 3. *H. jubatum* × *H. bulbosum*. Diakinesis: 13 pairs and 1 univalent, at least 7 pairs do not have chiasmata. Note pairing of chromosomes in adjacent cells.

FIG. 4. *H. jubatum* × *H. bulbosum*. Very early metaphase I: Ring in center is pseudo ring-bivalent without true chiasmata.

FIG. 5. *H. jubatum* × *H. bulbosum*. Very early metaphase I: Ring in center probably has one terminalized chiasma and one matrical attachment. Four unoriented *s–s* associations also shown.

FIG. 6. *H. jubatum* × *H. bulbosum*. Metaphase I: 4 bivalents and 19 univalents. Note *s–s* associations.

FIG. 7. *H. jubatum* × *H. bulbosum*. Metaphase I: 27 univalents.

FIG. 8. *H. jubatum* × *H. bulbosum*. Metaphase I: 4 bivalents and 19 univalents. Note shape of the *s–s* association at lower right.

PLATE I

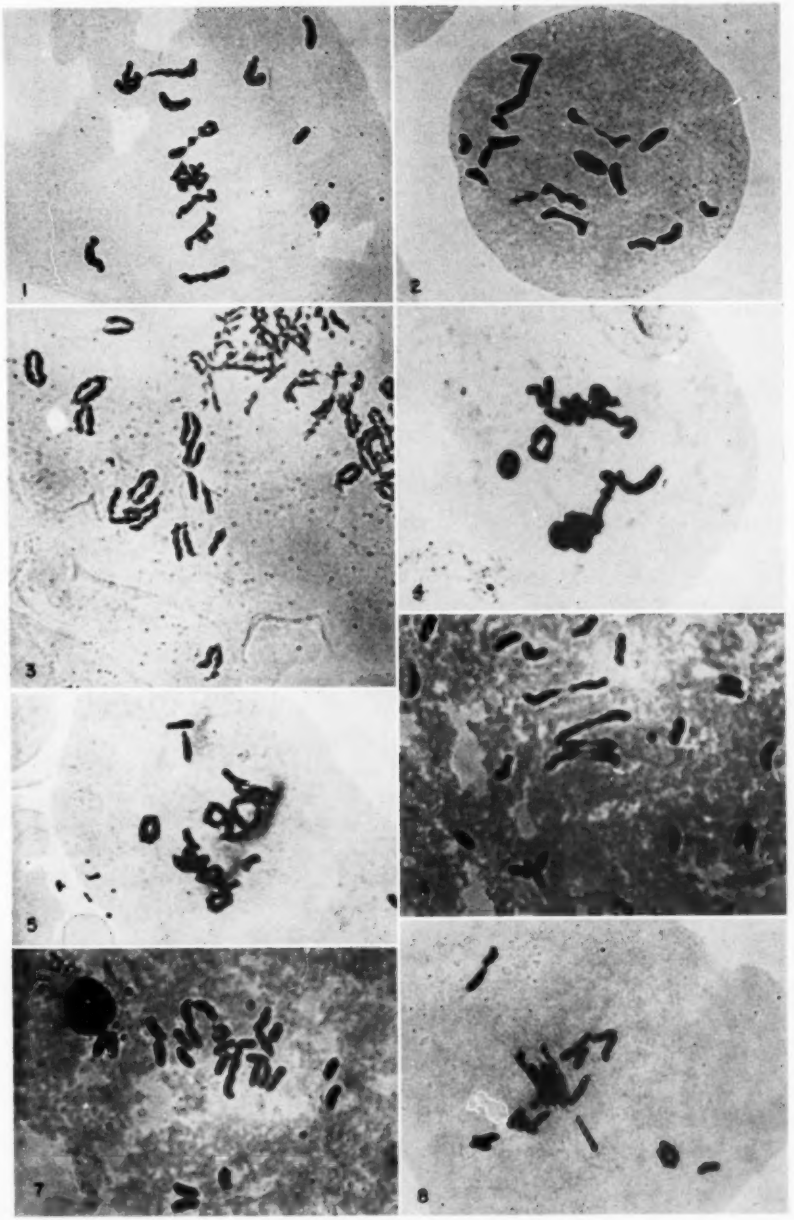

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TABLE IV

Summary of data concerning the formation of bivalents in five hybrids involving *Hordeum jubatum*

		1	2	3			4	5	6
No.	Hybrid	2n	Average number of bivalents	Range of number of bivalents per cell			Chiasmata per bivalent	Calculated* number of chromosomes potentially able to pair (p)	Expected number of pairable chromosomes
				Low	Mode	High			
33	*H. jubatum* (E) × *S. cereale* var. Antelope	21	5.9	3	6	8	1.59	12.69	14†
660	*H. jubatum* (S) × *S. cereale* var. Prolific	21	5.9	1	6	8	1.51	13.26	14†
727	*H. jubatum* (S) × *S. cereale* var. Prolific	21	4.4	0	4	7	1.28	12.85	14†
295	*H. jubatum* (S) × *H. bulbosum*	27	3.7	0	4	7	1.30	10.40	26
	H. jubatum (S) × *H. vulgare* var. Hannchen‡	21	1.2	0	0	4	1.22	3.65	14

*Calculated with Gaul's formula.
†Autosyndetic pairing of *Secale* chromosomes disregarded.
‡Only cells which had 21 chromosomes were considered.

Since the hybrids No. 660, 727, and 295 were grown in the same growth cabinet (with controlled light and temperature) the effect of the environment on the average number of bivalents formed (4, 17, 24) is minimal. It is concluded, therefore, that a genetically induced asynaptic effect prevented the formation of the maximum possible number of bivalents in the three hybrids having an average number of less than 5.9 bivalents per cell. The effect is relatively weak in *H. jubatum* × *S. cereale* (No. 727), but strong asynapsis occurred in the hybrid *H. jubatum* × *H. bulbosum* (No. 295) and in Kerber's *H. jubatum* × *H. vulgare* hybrid. A more or less similar variation in asynapsis was observed by Gaul in a large number of hybrids of *S. cereale* × *Agropyron intermedium* and *A. intermedium* × *Triticum aestivum* (4).

It will be of interest to apply Gaul's formula to these cytological data, so that some information can be obtained concerning the chiasma distributions in the pollen mother cells of these hybrids. Before commencing the analysis, however, some of the theoretical aspects of the formula will be considered.

This formula, $p = (X^2 + X - B)/((2X - B).Z)$, was developed by Gaul (4, 5) to calculate, from cytological data of plants showing genetically induced asynapsis, the number of chromosomes which are potentially able to pair (p) and to form bivalents if no asynapsis is present. For the application of this formula the following data are needed: the total number of chiasmata (X), the total number of chromosomes involved in the formation of bivalents and trivalents[4] (B), and the number of cells studied (Z). This formula is based on the premise that although the number of chiasmata may be reduced, the distribution of those that do occur in a nucleus will be at random. Each arm of a chromosome pair has thus an equal chance to receive zero or one

[4]The chromosomes involved in bivalent and trivalent formation are referred to as associated chromosomes.

chiasma. Consequently, each bivalent may have zero, one, or two chiasmata distributed in a random fashion. The two possible limits are thus: no chiasma (all univalents: minimum), or two chiasmata on each bivalent (all ring bivalents: maximum). The distribution of intermediate numbers of chiasmata (expressed as averages per cell) will follow a parabolic curve as shown in Fig. 9 (for extensive discussions see Gaul (5, 6)).

When the application of Gaul's formula leads to a good estimate of the maximum number of pairable chromosomes in an asynaptic plant, it is evident that the distribution of the chiasmata is random. But where Gaul's formula does not lead to a good approximation of the number of chromosomes capable of pairing, the possibility is introduced that another effect or effects have disturbed normal chiasma distribution. Gaul's formula can, therefore, be used to check the distributions of chiasmata in asynaptic plants.

For each hybrid the maximum pairing potential of the chromosomes (hereafter referred to as "calculated number" of pairable chromosomes) was calculated with the aid of Gaul's formula, and these values were included in Table IV (column 5). Column 6 shows the "Expected number" of pairable chromosomes for each hybrid, i.e., the maximum number of bivalents expected if no asynapsis was present. If the chiasma distributions of these five hybrids follow the random pattern the values in column 5 must be a close approximation of the corresponding values in column 6.

As can be seen in Table IV (columns 5 and 6), application of Gaul's formula gave reasonably good estimates of the pairing potentials of the chromosomes in the three *H. jubatum* × *S. cereale* hybrids. However, for the other two hybrids involving *H. bulbosum* and *H. vulgare* a calculated number of less than half of the expected number of pairable chromosomes was obtained. It is clear, therefore, that Gaul's formula is not applicable to these last two hybrids.

The question now arises: what caused the discrepancies in the calculated value of the maximum number of chromosomes potentially able to pair? Obviously, the distribution of the chiasmata in these two hybrids was not strictly random. A way to measure these discrepancies is again offered by Gaul's formula. This is done by applying the formula as follows: the total number of chiasmata (X) is assumed to be unknown, the expected number of pairable chromosomes (values in column 6, Table IV) is substituted for p, while for B and Z the observational data are maintained. In this way a new calculated value of X is obtained which is representative for the data of the hybrid concerned, if the chiasma distribution had occurred in a strictly random fashion. The difference between the calculated X and the observed X, compared in relation to the associated number of chromosomes observed, will indicate the extent and the nature of the discrepancies that occurred in the actual chiasma distributions.

This comparison is made in Table V. Here the data, necessary for the application of Gaul's formula for four hybrids, are included (columns 1, 2, 3, and 9). Columns 6, 7, and 8 show various values calculated from these data, which express the degrees of pairing. Row *a* contains the observed values representative for each of the hybrids and thus indicates the *actual* situation.

TABLE V

Observed and calculated data concerning chromosome behavior at metaphase I of four hybrids involving *H. jubatum*

			1	2	3	4	5	6	7	8	9
No.	Hybrid		Total no. of cells, *Z*	Total no. of associated chromosomes, *B*	Total no. of chiasmata, *X*	Total no. of chiasmata per cell	Associated chromosomes per cell	Chromosomes per chiasmata	Half chiasmata per chromosome	Chiasmata per bivalent	Calculated no. of pairable chromosomes (*p*)
33	*H. jubatum* × *S. cereale* var. Antelope	(a)	157	1868	1494	9.52	11.89	1.25	.80	1.59	12.69
		(b)	157	1868	*1346*	*8.57*	11.89	*1.39*	*.72*	*1.44*	(14)*
660	*H. jubatum* × *S. cereale* var. Prolific	(a)	221	2626	1985	8.98	11.88	1.32	.76	1.51	13.26
		(b)	221	2626	*1890*	*8.55*	11.88	*1.39*	*.72*	*1.44*	(14)*
727	*H. jubatum* × *S. cereale* var. Prolific	(a)	300	2654	1703	5.67	8.85	1.56	.64	1.28	12.84
		(b)	300	2654	*1652*	*5.51*	8.85	*1.61*	*.62*	*1.24*	(14)*
295	*H. jubatum* × *H. bulbosum*	(a)	127	944	615	4.84	7.43	1.53	.65	1.30	10.67
		(b)	127	944	*512*	*4.03*	7.43	*1.84*	*.54*	*1.08*	(26)*

*Expected number of pairable chromosomes used as the value of *p* in the recalculations.

Row *b* shows values treated and calculated as outlined above; the new, calculated X values and the correspondingly adjusted values in columns 4, 6, 7, and 8 are printed in italics. They thus represent the *hypothetical* situation that would exist if the chiasmata were distributed strictly at random over the paired chromosomes at meiosis of each of the four hybrids.

The figures in Table V show that although differences between the observed and calculated X-values occurred for each of the four hybrids, they were relatively small for the three *H. jubatum* × *S. cereale* hybrids (giving a reasonable estimate of p; see Table IV also). The difference concerning the *H. jubatum* × *H. bulbosum* hybrid, however, was relatively much higher, thus inducing a significant deviation in the estimate of the maximum number of pairable chromosomes. This trend is also clearly demonstrated in the values of columns 6, 7, and 8.

If the data of the hybrids are plotted in a graph, and locations representing the hybrids are examined in relation to their respective hypothetical curves, which are based on random distributions of the chiasmata over the paired chromosomes, the same pattern can be found. Figure 9 shows the ideal parabolic curves for hybrids having 14 and 26 chromosomes potentially able to pair, if the number of chiasmata that occur are distributed randomly. The positions of all five hybrids are indicated in the figure (for co-ordinates see columns 4 and 5, Table V). They are not located *on* the respective curves but are all lying off to the right. From this graph, and also from Table V, it can be seen that discrepancies in random chiasma distribution occurring in the

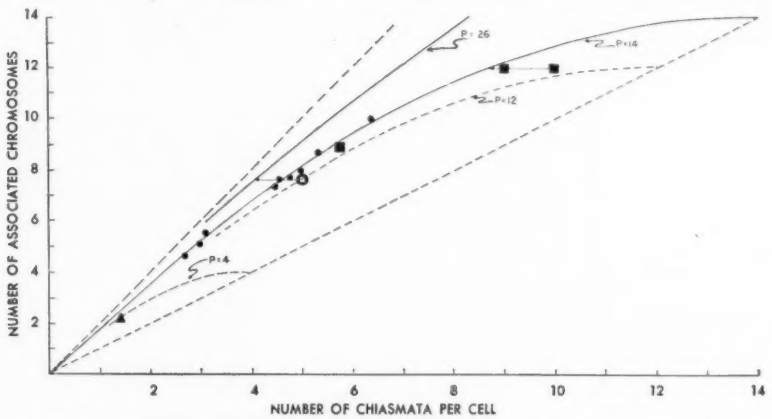

FIG. 9. A graphic representation of the ideal curve according to Gaul's formula (5) in which $p = (X^2 + X - B)/((2X - B).Z) = 14$, the maximum number of pairable chromosomes in the hybrids *H. jubatum* × *S. cereale* and *H. jubatum* × *H. vulgare*. The curve for the *H. jubatum* × *H. bulbosum* hybrid ($p = 26$) is partly shown. The positions of the hybrids involving *H. jubatum* and the asynaptic rye plants of Prakken (17) are indicated. As can be seen in this graph, the point representing the *H. jubatum* × *H. bulbosum* hybrid actually lies on a curve with $p = 12$. The point representing the *H. jubatum* × *H. vulgare* hybrid lies on a curve with $p = 4$ instead of that with $p = 14$. ■ *H. jubatum* × *S. cereale*, ▲ *H. jubatum* × *H. vulgare*, ○ *H. jubatum* × *H. bulbosum*, ● asynaptic *S. cereale*.

H. jubatum × *S. cereale* hybrids with higher averages of bivalent formation are less critical for a reasonable estimate of the *p*-value than in hybrids with a low degree of bivalent formation. The locations of the hybrids with regard to their respective hypothetical curve also indicate that in all hybrids the number of chiasmata was too high for the observed number of associated chromosomes per cell. It can be concluded that in the *H. jubatum* × *H. bulbosum* hybrid, as well as in the *H. jubatum* × *H. vulgare* hybrid, the chiasma distributions deviated significantly from random, in having more than the expected number of chiasmata. A similar conclusion may be drawn for the *H. jubatum* × *S. cereale* hybrids, although the deviations here were much less pronounced.

Since in the *H. jubatum* × *H. bulbosum* hybrid the number of trivalents formed was negligible, it can safely be said that the increase in chiasma frequencies beyond that expected must be due to the bivalents. This would thus imply that more ring bivalents were recorded than would be expected if the reduced number of chiasmata that occurred were distributed at random over the paired chromosomes. It is thus evident that one or more factors have been responsible for the high proportion of ring bivalents in these hybrids. Sampling error can be ruled out as a principal factor. If one compares the chiasma distributions of the hybrids with those of Prakken's asynaptic rye (Fig. 9), each of which had two completely homologous genomes (17), it becomes evident that sampling error played a minor role in causing the discrepancies as recorded for the hybrids.

In order to search for the cause of the above-mentioned discrepancies a detailed study of diakinesis and early metaphase cells of the *H. jubatum* × *H. bulbosum* hybrid was made. This study revealed that at the onset of metaphase I the chromosome pairs that did not form chiasmata either fell apart into univalents or remained attached to each other by matrical fusions or stickiness (Figs. 3, 4, 5, 6, 7, and 8). The chromosomes of the latter pairs often formed matrical attachments at both ends giving rise to oval-shaped chromosome associations that were frequently seen in cells at the transitional stage between diakinesis and metaphase I (Figs. 4 and 5). Further, it was observed that although these pairs lacked true chiasmata, they occasionally became co-oriented to form pseudo ring-bivalents. Such a co-oriented pseudo ring-bivalent is shown in Fig. 4; the cell in Fig. 5 includes a ring bivalent which has a chiasma in one arm and a matrix band in the other. Price made similar observations in diakinesis and metaphase I cells of *Secale montanum* (18).

At later stages of metaphase I of the hybrid *H. jubatum* × *H. bulbosum* the difference between true ring bivalents without visible chiasmata and pseudo ring-bivalents was difficult to determine. However, ring bivalents were often suspected of being pseudo bivalents.

Although pseudo ring-bivalents were difficult to recognize at metaphase I, additional observations on the metaphase cells indicated that they were very probably the origin of the discrepancies found in the chiasma distribution of the hybrid *H. jubatum* × *H. bulbosum*. The total bivalent population can be divided into a subpopulation of bivalents *with visible* chiasmata (59.4%)

and another subpopulation *without visible* chiasmata (40.6%). Application of Gaul's formula on the first subpopulation gave a p-value of 18.3, which is closer to the expected p-value of 26 than that of the total bivalent population ($p = 10.4$). Approximately 8% of the bivalents with chiasmata were scored as being ring bivalents. In contrast, the second subpopulation, consisting of bivalents without visible chiasmata, contained 82% ring bivalents.[5] As a result of the high incidence of chiasmata the p-value of this subpopulation was 3.6. It was, therefore, chiefly this subpopulation that greatly disturbed the random chiasma distribution of the total bivalent population. Since pseudo ring-bivalents were observed to occur at the transitional stage between diakinesis and metaphase I, it is very likely that they have contributed considerably to the high incidence of ring bivalents in this subpopulation and were the principal cause of the discrepancies in random chiasma distribution of the total bivalent population. However, ring bivalents with true chiasmata in both arm connections also probably occurred in addition to ring bivalents which had one true chiasma in one of the arms and a matrix strand in the other. It should be pointed out that these latter chromosome associations would have caused deviation from the random chiasma distribution similar to that caused by actual pseudo ring-bivalents.

From these considerations as well as from the cytological observations the conclusion can be drawn that in the hybrid *H. jubatum* × *H. bulbosum* the occurrence of pseudo ring-bivalents leads to a situation in which Gaul's formula cannot be legitimately applied.

The discrepancies in chiasma distribution in the *H. jubatum* × *S. cereale* hybrids, although not significant, cannot be due to sampling error alone. Some of the *H. jubatum* chromosomes are probably not completely homologous, a condition that may have slightly upset the random distribution of the chiasmata (see page 82, point 2). Occurrence of pseudo bivalents was noticed sometimes (Fig. 2), although their effect in disturbing the chiasma distributions was probably not great.

Discussion

Origin of the Asynapsis

In using cytological methods for studying genome relationships in plants, the average number of bivalents formed in the hybrid is used as a measure of the degree of homology existing between the genomes concerned. This method was developed by Kihara (8) in his extensive studies on the relationships between species of *Aegilops* and *Triticum*. However, since all gradations of genome relationships exist, and since chromosome pairing also may occur between partly homologous chromosomes, this method gives only an approximate identification of the genomes involved. This point has already been discussed by several writers (2, 9, 21, 22, 23).

On the other hand, similar genomes in a hybrid may be hindered in their bivalent formation by genetically induced failure of pachytene pairing or by

[5]These figures were obtained from the bivalent population of 46 of the 127 metaphase cells analyzed.

a reduction of chiasmata after chromosome pairing has occurred. Two of the three hybrids involving *Hordeum jubatum* discussed in this paper are examples of such genetically induced asynapsis. From the cytological data of the meioses of these hybrids, presented in the preceding sections, it is apparent that when genes of either *H. vulgare* or *H. bulbosum* are combined with those of *H. jubatum*, strong genetically induced asynapsis results.

However, Konzak *et al.* (10) and Morrison and Rajhathy (12) observed little variation in pairing behavior among the chromosomes of the hybrids of *H. vulgare* × *H. bulbosum*. Bivalents and multivalents occurred in fairly high frequencies, and the authors concluded that the *H. vulgare* chromosomes had a certain degree of homology with the chromosomes of *H. bulbosum*. The combination of *H. vulgare* and *H. bulbosum* genomes apparently did not produce any asynaptic effect. It may be concluded, therefore, that the genetically induced asynapsis in *H. jubatum* × *H. bulbosum* and *H. jubatum* × *H. vulgare* hybrids is the result of the combination of genes in the *H. jubatum* genomes with genes in the *H. bulbosum* and *H. vulgare* genomes. Interaction between genes of the genomes involved resulted in the reduction of the bivalent formation in each of these two hybrids. Since *H. bulbosum* is allogamous, and thus heterozygous, it is quite possible that if more hybrids involving *H. jubatum* were obtained and studied, a range from strong to weak asynapsis would be observed, as was noted by Gaul in his *T. aestivum* × *A. intermedium* hybrids (4). In order to obtain a similar range in bivalent and univalent formation, in hybrids with *H. vulgare*, which is autogamous, it might be necessary to combine *H. jubatum* with several *H. vulgare* varieties.

The weak asynapsis observed in the hybrid *H. jubatum* × *S. cereale* (No. 727) indicated that the rye genome may also slightly interfere with bivalent formation. Gaul (4) had a similar experience in his *S. cereale* × *A. intermedium* hybrids in which, however, both parental species were allogamous. In the present study it was more or less accepted that the average of 5.9 bivalents per cell represented the potential autosyndetic bivalent formation of the *H. jubatum* × *S. cereale* hybrids. This may not necessarily be so, since only three hybrids of this combination have been examined (27). Only a great number of hybrids of this combination, or still better, a polyhaploid of *H. jubatum*, can indicate the true autosyndetic capacities of the haploid set of the *H. jubatum* chromosomes.

Evaluation of Gaul's Formula

This study gave the opportunity to evaluate the applicability of Gaul's formula. From the data presented in this paper it appears that this formula, if applied to one or a few hybrids, must be employed with a certain degree of caution. Bivalent formation in an asynaptic individual may involve several factors other than reduction of the number of chiasmata alone. This may disturb the random chiasma distribution on which Gaul's formula is based. Had only the hybrids *H. jubatum* × *H. vulgare* (see also ref. 19) and *H. jubatum* × *H. bulbosum* been studied, observations on the formation of bivalents and the application of Gaul's formula on these data would have led to

the erroneous conclusion that *H. jubatum* is a true allopolyploid with little pairing affinity between its genomes. It is thus very desirable in such studies to investigate several interspecific and intergeneric combinations, in particular if self-fertilizing species are involved. If cross-fertilizing species are used several individuals of the same specific combination are probably necessary. Gaul has already pointed this out (5). However, if asynapsis is evident in a number of individuals, a study of the formation of bivalents followed by an application of Gaul's formula may be advantageous, even if the normal distribution of chiasmata is disturbed by secondary factors. With Gaul's formula disturbance of random chiasma distributions can be easily deduced. With additional observations the actual mechanism of these secondary effects may then be discovered. Although the applicability of this formula is limited to those cases in which chiasmata are distributed randomly over *completely paired chromosomes during pachytene*, it still will prove quite useful in other, more complicated cases to detect discrepancies and thus may lead to the recognition of other factors affecting the formation of bivalents in plants exhibiting induced asynapsis.

Factors Affecting Chiasma Distributions

Some of the factors that disturb chiasma distributions in plants having reduced chiasma formation and, therefore, result in erroneous estimates of p-values with Gaul's formula may include the following.

1. Non-homology of one chromosome arm (5, 6). Pairing will only take place between homologous arms.

2. Structural differences. It has been found in maize and *Drosophila*, that structural differences (as inversions, translocations, deficiencies, etc.) in certain chromosome pairs may suppress crossing-over in the pairs concerned (3, 25, 26). Similarly, in certain hybrids some chromosomes may pair in pachytene, although containing either structural differences or small non-homologous segments. It is conceivable that when such pairs occur *together with completely homologous pairs in asynaptic plants* (in which the number of chiasmata is thus reduced), they may form chiasmata less readily than the completely homologous ones. This would lead to a non-random chiasma distribution.

3. An additional factor which reduces prophase pairing. Chiasmata then will be distributed over fewer chromosome pairs and this would result in an upset of the random distribution, taking into account all chromosomes potentially able to pair.

4. A certain amount of pseudo bivalent formation.

The situations indicated in points 1, 2, and 3 should lead to a low estimate of the p-value. Pseudo bivalents (point 4), however, may induce high or low p-values.

Points 1 and 3 can be ruled out as factors disturbing the chiasma distribution of the *H. jubatum* × *H. bulbosum* hybrid, since the *H. bulbosum* parent is an autotetraploid and each chromosome has a homologue when in a haploid state (confirmed by good chromosome pairing observed at prophase I of the

hybrid). Further, the situation as mentioned in point 2 probably did not play a role either, although its possible existence as an influencing factor on the chiasma distribution cannot be entirely dismissed. The chiasma distributions of the *H. jubatum* chromosomes followed randomness rather well, as revealed in the *H. jubatum* × *S. cereale* hybrids. It is to be expected that a similar regular distribution would occur in the haploid set of the chromosomes of *H. bulbosum*. In the hybrid of these two species, which showed a strong reduction of chiasma frequencies, small structural differences or nonhomologous segments occurring between the *H. jubatum* chromosomes may have slightly favored the distribution of chiasmata among the completely homologous chromosomes of *H. bulbosum*. On the basis of cytological evidence, however, the major factor that caused the discrepancies in the chiasma distributions were chiefly due to the occurrence of pseudo ring-bivalents. The factor described in point 2 may have had a slight influence on the distributions recorded for the *H. jubatum* × *S. cereale* hybrids.

Origin of Pseudo Ring-Bivalents

In the section Analytical Studies, it was concluded that pseudo ring-bivalents, mistakenly interpreted as true bivalents, had upset the random chiasma distribution in the hybrid *H. jubatum* × *H. bulbosum*. Their occurrence was not discovered until the calculations with Gaul's formula and additional cytological observations were made. Obviously, the criterion that the bond of two arms is a terminalized chiasma was, in this material, a dubious one, since the difference between a true chiasma and a matrix attachment was difficult to establish. This may also be true in other material. However, application of Gaul's formula on the cytological data, including all observed "chiasmata", should then indicate their presence. If the calculated p-value is lower than expected, pseudo ring-bivalents may be a cause of this deviation; if the p-value is higher than expected a great frequency of pseudo rod-bivalents is very possible.

So far only pseudo bivalents (quasi bivalents) in rod shape have been reported (15, 28). Walters (28) suggested that these associations resulted from pairing at early prophase followed by matrical attachment of these paired chromosomes. She made the same suggestion for the origin of the "sticky univalents" which in contrast to the pseudo bivalents failed to orient at the metaphase plate. Person (16), confirmed by Riley and Chapman (20), showed in his studies on haploid *Triticum aestivum* that these "sticky univalents", in particular the *s–s* associations, were indeed the results of prophase pairing. These conclusions are valid for the *H. jubatum* × *H. bulbosum* hybrid, in which the *e–e* associations were also a direct result of prophase pairing. These kinds of associations may thus be indications of earlier chromosome pairing.

Östergren and Vigfusson (15) rejected the idea of the possible occurrence of associated univalents as a pairing relic. According to Östergren's theory of co-orientation by pulling (14) these associations should always form pseudo bivalents (15). On the basis of the cytological observations of Walters (28),

Person (16), Riley and Chapman (20), and the present studies, this viewpoint is untenable. Walters (28) suggested that the formation of pseudo bivalents is possibly dependent on the position of the association at prophase; if it is located near the equator it may become oriented; if located outside this region *s–s* or *e–e* associations may be the result.

This theory, however, is not very satisfactory. Chromosomes normally paired and with chiasmata will always become oriented (disregarding rare exceptions), although they are scattered throughout the cytoplasm at diakinesis. Obviously, no relationship between location and orientation exists. It is very unlikely, therefore, that such a location–orientation relationship would exist in cases of chromosome pairs matrically attached instead of by chiasmata. The following hypothesis is suggested as an alternative. When two chromosomes pair at zygotene they are long and completely despiralized. Chiasmata will be formed after completion of pairing. Spiralization then commences making the chromosome pair shorter and condensed. This contraction process is synchronized for each member of such a pair. The chiasmata will keep the homologous parts opposite each other. Consequently, the positions of the two centromeres in exact juxtaposition is strictly maintained. This condition leads to maximal chances of co-orientation after diakinesis (2, 14). However, if in an asynaptic plant two homologous chromosomes pair at zygotene and no true chiasmata are formed after completion of pairing, spiralization and condensation process can proceed independently so that the relative positions of homologous chromosome parts need not be maintained. As a result, the centromeres do not remain in exact juxtaposition. When matrix fusions occur at diakinesis, again binding the two chromosomes into a pair, the relative positions of the two centromeres are usually such that they cannot fulfill the conditions of co-orientation. In such circumstances these pairs will lead to *s–s*, *e–e*, and *e–s* (20) associations. However, when by chance the positions of the centromeres are exactly opposite each other, then the requirements of co-orientation are met and a pseudo rod- or pseudo ring-bivalent will be formed.

When asynapsis is due to reduced chiasma formation between paired chromosomes, it is to be expected that the more asynaptic the material, the greater the chances will be that associated univalents and pseudo bivalents will occur. The frequencies in which pseudo bivalents will be formed, however, may depend greatly on the material involved. Some individuals may exhibit only associated univalents, others may frequently show pseudo bivalents as well. Certain conditions prior to diakinesis may be responsible for this different expression. A possible genetic system controlling the formation of pseudo bivalents, as suggested by Walters (28), may actually influence these early meiotic prophase conditions.

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STUDIES ON THE WESTERN WOOD-ROTTING FUNGUS PORIA ZONATA BRES.[1]

R. L. GILBERTSON[2]

Abstract

Poria zonata Bres., a wood-rotting fungus known only from the western United States and British Columbia, is reported to be widely distributed within that area on recently fallen trees of *Abies grandis*, *A. concolor*, *A. lasiocarpa*, *Larix occidentalis*, *Pseudotsuga menziesii*, and *Tsuga heterophylla*. A description of the sporophores of the fungus, its cultural characteristics, and the white pocket rot caused by it are given.

Most species of the genus *Poria* known to occur in western North America are also reported from the eastern United States and Canada. A small number of well-defined species, however, are apparently confined in North America to the coniferous forest regions of the West. One of these is *Poria zonata* Bres., a fungus described in 1925 from specimens sent to Bresadola (2) from northern Idaho by J. R. Weir. Since that time very few collections have been made, and subsequent authors, including Baxter (1), Cooke and Shaw (3), Gilbertson (5), and Lowe (6), have added little to our knowledge of this species. This paper provides additional information on the morphology of *Poria zonata*, its distribution, substratum relationships, associated rot, and cultural characteristics.

The following description of the sporophores of *P. zonata* is based on studies of isotype material and 10 other collections over its known geographical range.

Poria zonata Bres. Mycologia 17:77. 1925.

Sporophores annual, resupinate, usually developing sporadically over a wide area on the bottom or sides of recently felled conifer logs, ivory-white when fresh, sometimes darkening to ivory-tan on drying; margin sterile, usually 0.5–1.0 cm wide, often concentrically zonate, fimbriate (Fig. 1); texture tough-fibrous; taste mild; pores angular, 1–2 per mm, with thick, entire dissepiments that become lacerate; context ivory, less than 0.5 mm thick, azonate, tough-fibrous; tube layer concolorous and continuous with the context, up to 4 mm thick.

Context hyphae hyaline in KOH solution, mostly thick-walled, rarely branched, rarely clamped, 2–7 μ in diameter, some thin-walled, rarely branched, clamped, 2–4 μ in diameter; tramal hyphae mostly thin-walled, rarely branched, clamped, 2–4 μ in diameter, some very slender, thick-walled hyphae intermixed; hymenial layer 14–15 μ thick; basidia clavate, four-spored, 11–12$\times$4–5 μ; cystidia very abundant, thick-walled, narrowly conical to subulate, capitately to almost entirely incrusted, 40–50$\times$9–11 μ; spores hyaline, smooth, cylindrical, mostly curved, 5–7.5$\times$2–2.5 μ (Fig. 8).

[1]Manuscript received August 10, 1959.
Contribution from the College of Forestry, University of Idaho, Moscow, Idaho.

[2]State University of New York College of Forestry, Syracuse, New York. A portion of this work was done while the author was Assistant Professor of Forestry at the University of Idaho.

Specimens Studied

On *Abies grandis* (Dougl.) Lindl.

J. R. Weir No. 71007; Priest River, Idaho; no date. (NYSCF)

J. R. Weir No. 15910; Priest River, Idaho; no date. (LOO); ISOTYPE

On *Abies concolor* (Gord. & Glend.) Lindl.

F. Hawksworth No. 151; Nelder Grove, Sierra National Forest, California; Dec. 27, 1950. (USFP)

On *Abies lasiocarpa* (Hook.) Nutt.

A. H. Smith Nos. 53424 and 53426; Papoose Creek, Seven Devils Mountains, Nez Perce National Forest, Idaho; Sept. 12, 1956. (UM)

On *Larix occidentalis* Nutt.

R. L. Gilbertson No. 1010; Binarch Creek, Kaniksu National Forest, Idaho; Oct. 12, 1955. (UIFP)

R. L. Gilbertson No. 1024; Granite Creek, Kaniksu National Forest, Idaho; Oct. 14, 1956. (UIFP)

On *Pseudotsuga menziesii* (Mirb.) Franco

C. R. Stillinger No. 4442; Granite Creek, Kaniksu National Forest, Idaho; Oct. 10, 1941. (LOO)

On *Tsuga heterophylla* (Raf.) Sarg.

J. R. Weir No. 4134; St. Joe National Forest, Idaho; no date. (LOO)

J. R. Weir No. 4141; Agassiz, British Columbia; on roots of a living tree; no date. (LOO)

R. L. Gilbertson No. 1025; Binarch Creek, Kaniksu National Forest, Idaho; Oct. 14, 1956. (UIFP)

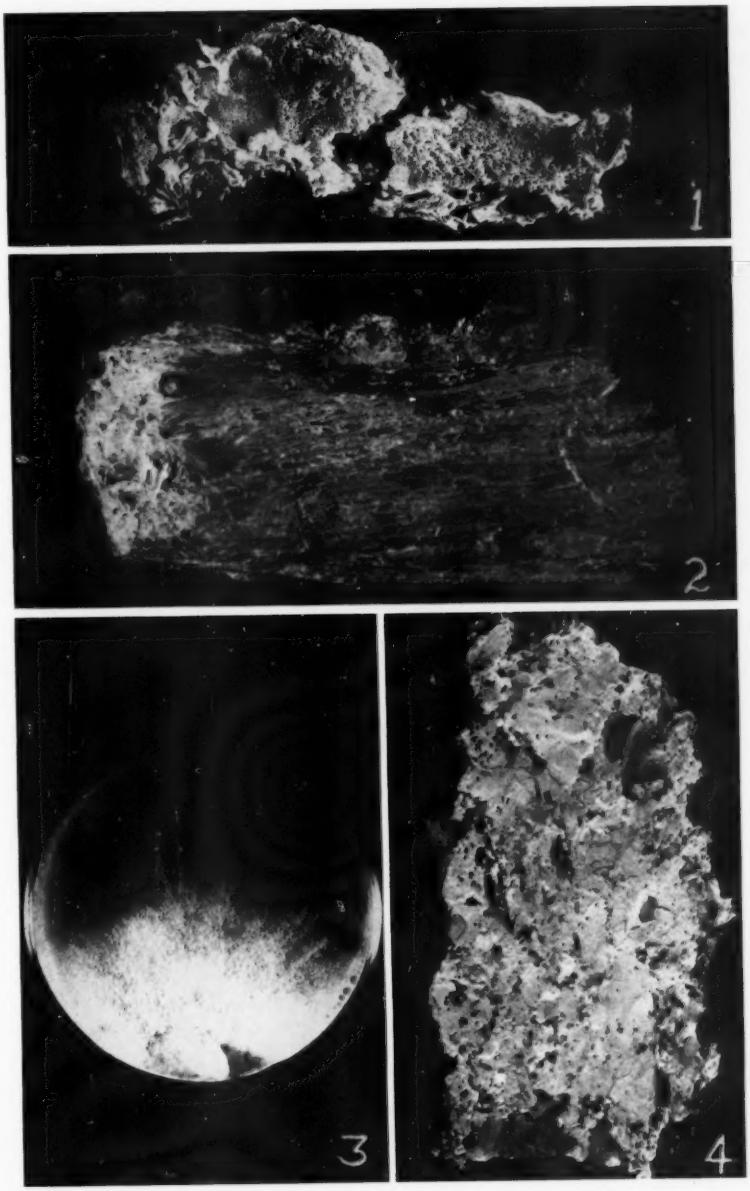
A. W. Slipp No. 1453; Nordman, Idaho; Dec. 10, 1942. (UIFP)

Sporophores of *P. zonata* are generally found on the bark of recently felled trees and are associated with an underlying white mycelial mat between the bark and wood of the substratum (Fig. 4). These mycelial mats are generally much more extensive than the associated sporophores. Conspicuous black flecks are present in the white mycelium. Hyphae of the mats are hyaline, thick-walled, apparently nonseptate, and 2–4 μ in diameter.

The macroscopic diagnostic characteristics of *P. zonata* are the large pores and the concentrically zonate, fimbriate sterile margin. Microscopically, the species is readily identified by the abundant, large, and incrusted cystidia. *Poria corticola* (Fries) Cooke is similar, but does not have the wide, zonate margin and is found mostly on hardwoods. It further differs microscopically in having thin-walled, septate hyphae, and broadly ellipsoid spores. *P. corticola* also has incrusted cystidia, but they are smaller than those of *P.*

FIGS. 1–4. Macroscopic features of *P. zonata*. 1. Fruiting body of *P. zonata* from western larch log (R. L. Gilbertson No. 1024); ×0.6. 2. Decayed wood taken from under fruiting bodies of *P. zonata* on western hemlock log (R. L. Gilbertson No. 1025); ×0.6. 3. Seven-day-old culture of *P. zonata* on malt agar medium (R. L. Gilbertson No. 1024). 4. Subcortical mycelial mat of *P. zonata* associated with fruiting bodies on western larch log (R. L. Gilbertson No. 1010). ×0.4

PLATE I



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zonata. Another species commonly found on conifer logs in the West, *Poria sinuosa* (Fries) Cooke, often has large pores and may resemble *P. zonata*. However, *P. sinuosa* may be readily differentiated by its smaller spores and lack of cystidia. Furthermore, the zonate and fimbriate sterile margin is not seen in that species. In his original description, Bresadola points out that "Dem *Poria radula* von Aussehen ganz gleich, aber, besonders durch Sporen, gut verschieden." This is apparently a reference to *Poria radula* (Pers.) Cooke, a species that also differs markedly in its pinkish, rhizomorphic sporophores.

Poria zonata was isolated in pure culture from spore prints obtained by attaching small pieces of freshly collected sporophores above the slant surface in tubes of malt agar media as illustrated by Davidson and Hinds (4). Cultural studies were carried out using the techniques of Nobles (7). A 2% malt agar medium was used, and cultures were incubated in the dark in petri dishes at 28° C. The fungus was also grown on standard gallic and tannic acid media and the presence or absence of diffusion zones noted 1 week after inoculation. An alcoholic solution of gum guaiac was also used to test for the presence of an extracellular oxidase according to Nobles (8).

Culture Examined

R. L. Gilbertson No. 1024, on western larch log, Granite Creek, Kaniksu National Forest, Idaho, Oct. 14, 1956. (Fig. 3)

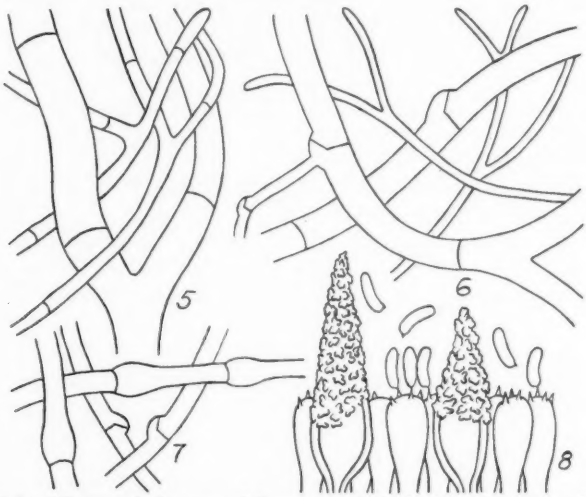
Growth rate rapid, plates covered in 1 week; advancing zone appressed, hyaline; white aerial mycelium developing 0.5–2.0 cm behind the advancing edge; aerial mycelium white to creamy-white, becoming woolly; reverse not darkened, apparently slightly bleached; odor fruity; reaction on gallic acid and tannic acid media positive, with a distinct diffusion zone developing on both media; a distinct dark-blue coloration developing rapidly where an alcoholic solution of gum guaiac is dropped on the mycelial mat.

Hyphae of advancing zone hyaline, with simple septa, frequently branched, 2–8 μ in diameter (Fig. 5); hyphae of aerial mycelium with simple septa and clamp connections (often on the same hypha), occasionally branched, 2.5–5.5 μ in diameter (Fig. 6); hyphae of the submerged mycelium as in the advancing zone but with occasional clamp connections, often swollen conspicuously at the septa (Fig. 7); asexual spores not observed on any part of the mycelium.

Cultures of *P. zonata* growing in tubes of malt agar media develop areas of yellowish-brown, crustlike mycelium after several weeks. No coloration has been observed in cultures growing in petri dishes for periods up to 8 weeks.

P. zonata has been generally reported on coniferous logs and slash, never on hardwood substrata. Weir's collection from the roots of living hemlock in British Columbia is the only known report of the fungus on a living tree. Dissections of logs bearing *P. zonata* sporophores show that the associated decay is of the white rot type. Standard laboratory decay tests with *Poria zonata* on blocks of western larch and western hemlock wood have been carried out, and these show the development of a white rot. The previously described cultural work with gallic and tannic acid media and gum guaiac supports this

observation. Wood in the incipient stages of decay shows a slight pinkish or reddish discoloration. In the advanced stage of decay, the rotted wood has the characteristics of a poorly defined white pocket rot or a white mottled rot. Conspicuous blackish flecks are abundant in the "pockets" of more advanced decay and run parallel to the grain of the wood. Conspicuous whitish pockets of mycelium that run perpendicular to the grain are seen in all substrata and are particularly abundant in hemlock. These pockets evidently result from shrinkage of the wood and development of mycelium in the cracks. The decayed wood also develops a "ring shake" structure and tends to separate readily along the annual rings (Fig. 2). Because of the blackish flecks and poorly defined white pockets, the rot may be easily confused with that of *Fomes annosus* (Fries) Cooke, a common fungus in coniferous logs and slash in the cedar–hemlock forests of northern Idaho.



FIGS. 5–8. Microscopic features of *P. zonata*. 5–7. Hyphae of *P. zonata* in culture: 5, hyphae from advancing zone; 6, hyphae from aerial mycelium; 7, hyphae from submerged mycelium. 8. Hymenium of *P. zonata* showing incrusted cystidia, basidia, and spores. ×1000

From these studies it appears that *P. zonata* is a widely distributed fungus in the West. It is responsible for a white pocket rot of recently felled logs of a number of species of western conifers, and the rapidity with which the decay develops indicates that it may contribute to losses in salvable timber. The presence of the extensive subcortical mycelial mat suggests that it may spread quickly in a log by the rapid development of this mat in the cambial region and the subsequent penetration of hyphae into the wood over a large surface area from this mat.

The generous loan of specimens or use of herbarium facilities of the University of Idaho Forest Pathology Herbarium (UIFP), the State University of New York College of Forestry (NYSCF), the L. O. Overholts Herbarium of

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NOTES TO CONTRIBUTORS

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